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PROCEEDINGS OF THE ROYAL SOCIETY.

SECTION B.—BIOLOGICAL SCIENCES.

The Energy Liberated by an Isolated Muscle during the Performance of Work.

By W. HARTREE and A. V. HILL. F.R.S.

(Received October 4, 1928.)

(From the Physiological Laboratories, Cambridge, and University College, London.)

The investigations of Fenn (1) (2) established the fundamental fact that the total energy liberated by a stimulated muscle depends upon the amount of work which it does, and also upon the manner, and the phase of contraction, in which that work is performed. The extreme importance of Fenn's results, in any theory of muscular contraction, has long made a further study of the subject desirable. This, and the development of various improvements in technique, particularly of the ergometer of Levin and Wyman (3) which we used in the experiments described in a recent paper (9), have caused us during the last 18 months to make the experiments described below; these have led us to a new standpoint in relation to the thermodynamics of muscle.

We had no difficulty in confirming Fenn's most important conclusion, that the performance of work by a muscle causes a considerable increase in the total energy liberated, *but with the provision that the contraction is of a tetanic nature*. Most unexpectedly, however, we found that *in a single twitch the total energy is the same whether work be done or not*.^{*} This point was clearly of great theoretical significance. One statement of Fenn's, moreover, we were unable to verify, viz., that work done during the phase of relaxation causes a

^{*} In Fenn's first paper (see for example his figs. 2 and 5 and his Tables II and III) he found the same increase of energy with work in the case of a twitch as in that of a short tetanus. We cannot reconcile this with our results (see Tables I and IA).

decrease in the total energy set free.* Finding it impossible to explain this disagreement, and wishing to confirm our observation that the energy liberated in a twitch is unaffected by work done, we made a further series of 34 complete experiments, of which 23 employed the thermopile wound on a silver-frame described recently (11). By using this thermopile we hoped to eliminate any possibility of temperature differences along the length of the shortening muscle. The second series of experiments confirmed the first.

All the observations here recorded employed the sartorius muscles of *Rana temp.* or *Rana esc.*, and the Levin-Wyman ergometer. The thermopiles were various; the results obtained with the silver-frame instrument are denoted by S. The methods used have been described in previous papers.

Various experiments were performed with semi-membranosus muscles, but with different and inconsistent results. To secure intelligible and reliable data from the myothermic method, especially in such experiments as these where the muscle is allowed to shorten, a very thin muscle must be used, otherwise (a) the oxygen supply will be inadequate and after a time the muscle will contract differently at different spots; (b) the resting heat production, being proportional to the mass of the muscle, will be large enough to cause appreciable temperature differences between points on the thermopile and points not on it; and (c) temperature equalisation and heat conduction will be so slow that the galvanometer deflection will not represent a true average rise of temperature in the muscle. The muscle also should have its fibres as nearly parallel as possible and should be approximately of uniform cross-section.

Recently Prof. Bouckaert, performing similar experiments at University College on the biceps cruris of a tortoise, obtained anomalous, and obviously fallacious, results with thick muscles from large animals, but apparently reliable and intelligible results with thin muscles from small ones. In the case of the semi-membranosus, in our experiments, the isometric energy appeared to be greater than the energy when work was done. The results, however, were irregular and inconsistent. The heat was probably not produced uniformly throughout the substance of the muscle and the galvanometer deflection therefore was not proportional to the total heat. It seems extremely unlikely that the fibres of the one muscle differ in fundamental behaviour from those of the other, and all our experience suggests that results obtained with the sartorius of small frogs are more reliable and consistent than any others. The myothermic method, unfortunately, cannot be made a routine applicable to any kind of muscle at will; and we would take this opportunity of urging that those who employ it should use the thinnest and most uniform muscles available. Otherwise errors may arise which only long experience can detect.

The results of Table I show that in the case of a twitch the performance of work has no effect on the amount of energy set free.

* Fenn did not always find this effect. On p. 391 of his second paper he noted that the working heat was less than the isometric heat in only 11 out of 17 cases, and in his summary the statement was guarded by the word "usually."

Table I.

The Absence of Effect of the Performance of Work on the Energy Liberated in a Muscle Twitch.

First Series.

Experiment of 17.2.28, at 0° C. in O₂. A peculiarly good experiment and consistent in every respect.

I. 3 isometric twitches, 3 with nearly maximum work, and 3 isometric again.

Mean isometric heat	40 grm. cm.
Mean working heat	31½ „
Mean work	9½ „
Mean working energy	40½ „

Excess energy, working, + 2 per cent.

II. Repeated. 5 isometric twitches with photographic records: 6 working, ditto; 5 isometric, ditto.

Mean isometric heat	42½ grm. cm.
Mean working heat	33½ „
Mean work	9½ „
Mean working energy	43 „

Excess energy, working, + 1 per cent.

Experiment of 11.2.28, at 13° C. in O₂. Scale readings only.

Excess energy, working, — 4 per cent.

Experiment of 15.2.28, at 0° C. in O₂.

I. Scale readings only. Excess energy, working, + 5.5 per cent.

II. Photographic records. Excess energy, working, 0 per cent.

Experiment of 1.3.28, at 0° C. in O₂. Photographic records.

Excess energy, working, + 4 per cent.

Experiment of 2.3.28, at 0° C. in O₂. Photographic records.

Excess energy, working, 0 per cent.

Experiment of 5.3.28, at 0° C. in O₂.

Excess energy, working, — 2 per cent.

Second Series. (All at 0° C. in O₂.)

(Where several figures are given, each represents the mean of a separate group.)

Experiment of 25.7.28. S.—Mean of 3 sets of observations, each with “reverse.”

Work 10 grm. cm.; energy 35 grm. cm.; isometric heat 31½ grm. cm.

Excess energy, working, + 11 per cent.

Experiment of 26.7.28. S.—Excess energy, working, + 5 per cent.

Experiment of 17.8.28. S.—

Excess energy, working (1)	+ 2½ per cent.
„ „ (2)	+ 13 „
„ „ (3)	+ 15 „
„ „ (4)	0 „

Mean + 7½ per cent.

Table I—(continued).

Experiment of 1.8.28.—

Excess energy, working (1)	+ 6 per cent.
„ „ (2)	+ 6 „
„ „ (3)	+ 11½ „
„ „ (4)	+ 10 „
„ „ (5)	+ 9 „
Mean	+ 8½ per cent.

*Experiment of 8.8.28.—Excess energy, working, + 1 per cent.**Experiment of 14.8.28.—*

Excess energy, working (1)	— 1½ per cent.
„ „ (2)	— 2½ „
„ „ (3)	0 „
Mean	— 1½ per cent.

Experiment of 15.8.28.—

Excess energy, working (1)	+ 1 per cent.
„ „ (2)	+ 1 „
Mean	+ 1 per cent.

*Experiment of 22.8.28.—Excess energy, working, — 7½ per cent.**Experiment of 24.8.28.—*

Excess energy, working (1)	— 4½ per cent.
„ „ (2)	+ 1 „
Mean	— 2 per cent.

In the first series of Table I the average value of the excess energy in the working case was less than 1 per cent., with an average “initial” efficiency in the twitch (data not given in the table) of 22 per cent. (not quite maximal). In the second series the average value* of the excess energy was about 2½

* The second series of Table I includes several experiments showing an appreciable positive excess energy in the working case. The effect of these on the average is not large. The positive values, however, were not due to chance, but to some consistent cause, as is seen particularly in the experiment of 1.8.28. Each value given, moreover, is the mean of a considerable group of separate observations. We are inclined to attribute these inconsistencies to slight irregularities in the face of the thermopile. Under high tension the muscle might tend to draw away from the hollows, and to rest only on the crests, of these irregularities. Consequently, under tension it would make less perfect thermal contact with the hot-junctions, and the recorded deflection for a given rise of temperature would be less. Thus there might be a tendency, greater if the thermopile were less perfectly plane, for the isometric heat to appear smaller than the working heat: in the isometric case the muscle, under high tension, would stretch from crest to crest, and make less perfect contact between; in the working case, under smaller tension, and thickening as it shortened, it would maintain a better contact. The irregularities, of course, are slight. They were

per cent., with an average efficiency of $23\frac{1}{2}$ per cent. In nearly all these experiments (and in many others) observations were made also on tetanic contractions, in which case there is always a considerable excess energy in the working case. For example, for comparison with the second series of Table I, the following results may be quoted. See also Tables III and VI below.

Table IA.

The Effect of the Performance of Work on the Energy Liberated in a Tetanus.

The "excess energy working" is expressed as a percentage of the "isometric energy."

Experiment of 25.7.28. S.—0.1 second tetanus, excess working, 33 per cent.; 0.4 second tetanus, excess working, 40 per cent.

Experiment of 26.7.28. S.—0.1 second, 37 per cent.; 0.4 second, 53 per cent.

Experiment of 17.8.28. S.—0.3 second, 48 per cent.

Experiment of 1.8.28. —0.3 second, 27 per cent.

Experiment of 8.8.28. —0.3 second, $23\frac{1}{2}$ per cent

Experiment of 14.8.28. —0.3 second, 19 per cent.

Experiment of 22.8.28. —0.3 second, 16 per cent.

† *Experiment of 11.7.28.* S. —0.1 second, 20 per cent.

† *Experiment of 13.7.28.* S.—0.1 second, 27 per cent.; 0.4 second, 51 per cent.

† *Experiment of 11.7.28.* S.—0.1 second, 29 per cent.

† No observations on twitches, so no record in Table I.

In a short tetanic contraction, therefore, the energy liberated in the working case was, on the average, about 30 per cent. greater than that of the isometric contraction; about 23 per cent. of the whole energy in the working case is excess due to working. In these particular contractions the efficiency averaged 23 per cent., so that the excess energy was equal to the work done. This relation was found by Fenn, but we shall show below that it is only a special case determined by the choice of the duration of stimulus. There is no doubt, however, of the existence of the Fenn effect in tetanic contractions observed in the same experiments as, and under conditions identical with, the twitches which failed to show it. Just as large a proportion of total energy was liberated as work in the case of the twitch as in that of the tetanus; the efficiency was just the same. In the twitch, however, the excess energy due to work was absent, or at any rate so small that, on the average, it remained an insignificant fraction of the work done.

To obtain, if possible, some further information on the absence of the Fenn considerably greater, however, in Fenn's thermopiles than in those we have used. It may be that the effect occasionally observed by ourselves, and consistently found by Fenn, is to be attributed to the same technical cause, a difference between the degrees of contact of muscle with thermopile in the two cases, viz., isometric and working twitch. We have noticed that the larger "excesses" in the working case arose when the muscle had a greater initial tension.

effect in the case of a twitch, in four of the experiments of Table I photographic records were made, and a complete analysis carried out of the time-distribution of the heat, for both cases, isometric and working. The results are of peculiar interest, in showing that the work done is simply subtracted from the heat liberated in relaxation: see Table II.

Table II.

The Effect of the Performance of Work on the Relaxation Heat in a Twitch.

	Contraction heat.	Relaxation heat.	Work.
<i>Experiment of 17.2.28—</i>			
Isometric	27½	15	—
Working	27½	5½	9½
<i>Experiment of 15.2.28—</i>			
Isometric	29½	15	—
Working	29½	5	10
<i>Experiment of 1.3.28—</i>			
Isometric	34	17	—
Working	32½	6	14½
<i>Experiment of 2.3.28—</i>			
Isometric	30	15	—
Working	31	6	8

We may conclude that a muscle undergoing a twitch behaves exactly as a simple viscous-elastic body, the total energy liberated being independent of the work done; the amount of the mechanical work is subtracted quantitatively from the heat liberated in relaxation. These observations confirm the view that the heat liberated in relaxation represents mechanical potential energy which is dissipated when the contraction passes off.

Even a slight increase in the duration of the stimulus causes an appreciable excess heat to appear in the working case. For example in the experiment of 14.2.28 at 13° C., in which the excess energy, working, in the twitch was —4 per cent., the following results were obtained for a stimulus of 0·05 second:—

	gram. cm.
Isometric heat	114
Working heat	98½
Work	39
Working energy	137½
Excess energy, working, 20 per cent.	

Again, in the experiment of 2.3.28 at 0° C., where the excess energy, working, in the twitch was 0 per cent., for a stimulus of 0.05 second we found :—

	gram. cm.
Isometric heat	53
Working heat	48
Work.....	12
Working energy	60
Excess energy, working, 13 per cent.	

The experiment of 5.3.28 at 0° C., for a twitch gave an excess energy, working, of --2 per cent., for a stimulus of 0.02 second of 7 per cent., and for a stimulus of 0.05 second of 12 per cent. The effect has been investigated in greater detail for longer stimuli. Some typical experiments are given in Table III.

Table III.

Effect of Duration of Stimulus on the Excess Energy Due to the Performance of Work.

Experiment of 27.4.27, at 0° C. with full "reverse," mean values given, records shown in fig. 1.

Heats, etc., in gram. cm. :—

Duration, seconds	0.06	0.25	0.50	0.75	1.0
Isometric heat	61	75	90	99	115
Working heat	52	76	97	116	134
Work	18	27	37	46	55
Working energy	70	103	133	163	189
Excess energy	9	28	43	64	74

Experiment of 28.4.27, at 0° C. with full "reverse," mean values given :—

Duration, seconds	0.06	0.25	0.5	0.75	1.0
Isometric heat	72	97	123	146	166
Working heat	56	89	117	141	160
Work	18	28	39	48	54
Working energy	74	118	156	188	214
Excess energy	2	21	33	42	48

Experiment of 29.4.27, at 0° C. with full "reverse," mean values given :—

Duration, seconds	0.06	0.25	0.6	1.0
Isometric heat	60	87	113	145
Working heat	52	82	118	149
Work	13	22	35½	49
Working energy	65	104	153	198
Excess energy	5	17	40	53

It is clear that as the duration of the stimulus increases the *heat* in the working case approximates to the *heat* in the isometric case, sometimes even surpassing it as in the first experiment of Table III. Fenn, finding that (with

the durations of stimulus which he employed) the working heat and the isometric heat were approximately equal, concluded, quite naturally, that extra energy is mobilised to meet the demands of the working muscle. The same demands, however, would presumably exist in the case of a single twitch, but apparently they are not met by an extra mobilisation of energy. Some other explanation, therefore, must be found.

Various experiments have been performed at 0° C., with stimuli of 0.2 and 0.4 second, and full analysis of photographic records, to find whether, in the case of such longer stimuli, the fact established in Table II still holds, viz., that work done causes an equivalent decrease in relaxation heat. The results of nine such experiments are given in Table IV.

Table IV.

To Compare Relaxation Heat, Isometric and Working, and to show Excess Energy in Relaxation to Work.

Date of experiment.	29.11.27.	1.12.27.	3.12.27.	7.12.27.	9.12.27.	13.12.27.	14.12.27.	16.12.27.	16.12.27.	Mean.
Stimulus duration, seconds..	0.4	0.2	0.4	0.4	0.4	0.4	0.4	0.4	0.2	—
Isometric—										
(a) Total initial heat, grm. cm.	84	82	96	75	99	68	98½	125	93	91
(b) Relaxation heat grm. cm.	42	38	34½	30½	40	29½	45½	51	45	39½
Working—										
(c) Total initial heat, grm. cm.	76	69	95	68½	85	67	95	123	85	85
(d) Work, grm. cm.	22	16½	34	20	21	28	29	32½	19½	25
(e) Total energy = (c + d) ..	98	85½	129	88½	106	95	124	155½	104½	109½
(f) Relaxation heat, grm. cm.	15½	17	5½	18	16	13	33	20	17½	17
(g) Efficiency = d/e	22½	19	26	23	20	29	23	21	19	22½
(h) $\frac{e - a}{d}$ Excess energy = work	0.64	0.22	0.97	0.68	0.33	0.97	0.88	0.94	0.59	0.69
(i) b - d - f	4½	4½	-5	-7½	3	-11½	-16½	-1½	8	-2½

Notes.

- (1) On the average, relaxation heat, isometric, = 43 per cent. total initial energy.
- (2) Line (i) shows that, on the average, approximately, (Relaxation, heat, isometric) = (Relaxation heat, working) + (work).
- (3) The efficiency in line (g) is given as a per cent. It was not necessarily a maximum; its average value was 22½ per cent. in these experiments as compared with the average value for the maximum efficiency—given in the previous paper—of 26 per cent.
- (4) Mechanical records of experiment of 14.12.27 are shown in fig. 1.



FIG. 1.—Mechanical records with Levin-Wyman ergometer.

(A) *Experiment of 27.4.27, Table III.* In order, starting from right bottom corner : 0.06 second, tetanus, 5 working, 2 isometric ; 0.25 second, 3 working, 2 isometric ; 0.50 second, 3 working, 2 isometric ; 0.75 second, 3 working, 2 isometric ; 1.0 second, 4 working, 2 isometric ; 0.75 second, 3 working, 2 isometric ; 0.5 second, 3 working, 2 isometric ; 0.25 second, 3 working, 2 isometric ; 0.06 second, 4 working, 2 isometric. Shortening in direction of arrows.

(B) *Experiment of 14.12.27, Table IV.* 0.4 second, tetanus. In order starting from right-hand bottom corner : 6 working, 6 isometric, 6 working, 6 isometric, all with photographic records for analysis. The experiments marked with an asterisk, which were the first of their respective series, were neglected. The remaining 10 records of each kind, working and isometric, were analysed and are averaged in Table IV.

It is seen that although, in individual experiments (line (i)) the relation does not hold exactly, there is, on the average, very little difference between relaxation heat in the isometric case, and relaxation heat plus work in the working case. The average value of (relaxation heat, isometric) is $39\frac{1}{2}$ grm. cm., of (relaxation heat, working) 25 grm. cm., and of work 17 grm. cm. The former differs from the sum of the latter by only 6 per cent., an insignificant amount. The individual variations (line (i)) are probably due to experimental

error and to ambiguity in calculation. The chief difficulty in such experiments and analyses as these is the decision of how much heat is to be ascribed to relaxation; the division between the two phases of heat-production, viz., contraction and relaxation respectively, is not always very sharp, and considerable error may arise when the analysis has to be carried out twice (for isometric and working) and the results subtracted. There is no reason, however, that we know of, why consistent (as distinguished from random) errors should arise, so that the average values should be reasonably near the truth. They show that the diminution in relaxation heat, due to work, is very nearly equal to that work. Thus the relation found in Table II is true not only for twitches but for short tetanic contractions.

With stimuli as short as 0.2 and 0.4 second at 0° C. the excess energy in the experiments of Table IV, though considerable, was not as great as the work done; the average value of the ratio $\frac{\text{excess energy}}{\text{work}}$ was 0.69. This excess energy is about equal to the increase in the heat-production during the contraction phase when work is done. The "contraction-heat," as distinguished from the "relaxation-heat," has in Table IV the following mean values: isometric $51\frac{1}{2}$, working 68: difference $16\frac{1}{2}$. The total energy has the values: isometric 91, working $109\frac{1}{2}$: difference $17\frac{1}{2}$, practically the same. Thus the following reciprocal relations exist:—

- (1) *Work diminishes relaxation heat;*
- (2) *Excess energy due to work appears as an increment in contraction heat.*

Of these (1) is easily intelligible if we regard work and relaxation heat as being derived from the same source, viz., mechanical potential energy; (2) is not so obviously intelligible, until we remember that it is really a necessary consequence of (1). Let C_i and R_i be contraction and relaxation heat respectively in the isometric case, C_w and R_w the same quantities in the working case, and W the work. Then (1) tells us that $R_i - R_w = W$. Now the excess energy due to work is equal to (total energy working) — (total energy isometric) = $(C_w + R_w + W) - (C_i + R_i)$, which, since $R_w + W = R_i$ is equal to $(C_w - C_i)$, the increment in the contraction heat. Thus, if relation (1) be true, its reciprocal relation (2) must be true also, and we are left only with the difficulty of explaining why, when work is done in a tetanic contraction (but not in a twitch), there is in fact an excess of total energy mobilised.

In a previous paper by Hartree (4) it was shown that in a contraction resulting from a short tetanus (0.1 second at 0° C.), in which a weight was lifted and

held up and not allowed to bear on the muscle during relaxation, the energy liberated in the form of heat was the same as in the isometric case, an extra amount of energy being mobilised equal to the work done. An analysis of the heat-production showed that, as in the present case, there was an excess of heat in the first phase (contraction) and a deficit of heat in the second phase (relaxation), these being rather less than the work performed. In the previous investigation work equal to 21 per cent. of the total energy was done, but only in the contraction phase; in the present one it was done mainly during contraction but partly also during relaxation. As in the previous paper (4) we find that the early heat-production is considerably greater in the working case.

The fact that the performance of work causes an equivalent diminution in the relaxation heat supports the idea that both have the same origin, viz., the mechanical potential energy present in the active contracting muscle. The only evidence we possess of the magnitude of this potential energy is that derived from the area of the tension-length curve as defined in (5). It is, however, by no means certain that this area does, in reality, represent the potential energy. The greatest work which a frog's muscle can do, in a contraction of short duration, is never more than about 25 per cent. of the tension-length area, and although it is clear that irreversible processes are involved in an actual shortening of a muscle, it is not so readily believed that 75 per cent. of the potential energy is of necessity degraded into heat owing to these irreversible processes. In a number of experiments we have compared R , the heat liberated during relaxation (i) with A , the area of the tension-length curve, and (ii) with the product Tl , where T is the full tension developed at the initial length l . The muscle was stimulated at a length determined by an initial load of 2 or 3 grammes, and during the course of the experiment observations were made of the tension developed at various shorter lengths, in the manner described by one of us (5). The analysis of photographic records of the isometric heat was carried out as usual, to give the heat R liberated during relaxation, which was then compared with the area A of the tension-length curve, or with the product Tl .

Table V.

The Relation between Relaxation Heat and Area of Tension-length Curve.

The symbol R is used to denote the relaxation heat, A to denote the area of the tension-length curve.

Experiment of 15.10.27.—0° C. in O₂.

$\frac{1}{2}$ second, tetanus, $R/Tl = 0.104$.

$\frac{1}{2}$ second, tetanus, $R/Tl = 0.113$.

1 second, tetanus, $R/Tl = 0.120$.

Experiment of 28.10.27.—0° C. in O₂, $\frac{1}{2}$ second, tetanus, $R/Tl = 0.109$.

$R/A = 0.53$.

Experiment of 29.10.27.—0° C. in O₂, $\frac{1}{2}$ second, tetanus, $R/Tl = 0.113$.

$R/A = 0.53$.

Experiment of 31.10.27.—0° C. in air, 0.6 second, tetanus, $R/Tl = 0.101$.

$R/A = 0.61$.

Experiment of 2.11.27.—0° C. in air, 0.8 second, tetanus, $R/Tl = 0.130$.

$R/A = 0.73$.

Experiment of 5.11.27.—0° C. in air, 1.0 second, tetanus, $R/Tl = 0.159$.

Experiment of 7.11.27.—0° C. in O₂—

0.2 second, tetanus, $R/Tl = 0.102$, $R/A = 0.50$.

1.0 second, tetanus, $R/Tl = 0.123$.

0.2 second, tetanus, $R/Tl = 0.116$.

In the five cases where R/A was determined its mean value is 0.58. The mean value of R/Tl is 0.117; according to A. V. Hill (5, p. 258) the mean value of A/Tl is 0.244, from which R/A is calculated as 0.48. Taking 0.53 as a mean value for R/A we must conclude, either:—

- (a) That the relaxation heat represents only a fraction of the mechanical potential energy which disappears in relaxation; or
- (b) That the mechanical potential energy is considerably less than the area of the tension-length curve.

If (a) be the case we must regard a part of the mechanical potential energy (about 50 per cent.) as being restored to the muscle during relaxation, in some latent physical or chemical form, instead of being dissipated as heat. This suggestion was made by Wyman (7) and discussed by one of us (8). A difficulty in accepting it is that apparently only about half of the mechanical potential energy is so retained, half being degraded into thermal agitation; thus, if work W be done, the diminution in relaxation heat should be, not W as we actually find it, but $\frac{1}{2}W$. The hypothesis appeared to have the advantage that it affords some kind of explanation of the excess energy associated with the performance of work; if work were done, the potential energy would be correspondingly diminished, and there would be less energy to restore in latent

form in relaxation ; consequently the net energy liberated would be greater. The advantage is, in fact, illusory since the hypothesis should apply equally to the case of a twitch, where, however, excess energy due to the performance of work does not appear. It may still be the case that the relaxation heat does not represent the whole of the mechanical potential energy, but, if so, some other endothermic process unknown must be proceeding during relaxation, diminishing the heat due to the degradation of potential energy.

The possibility (b) is more hopeful, viz., that the mechanical potential energy is considerably less than the area of the tension-length curve. We know from the work of Fick. and from that of Levin and Wyman, that a muscle stimulated continuously and allowed to shorten slowly will do work equal to the full area of its tension-length curve. This indeed is necessary if the full tension capable of being exerted at any length in a slow shortening is a function only of that length, and does not depend on what has happened previously—disregarding of course the onset of fatigue. When, however, a muscle shortens more rapidly it may well be the case that work done during shortening somehow influences the tension which it can exert at shorter lengths. A simple analogy may make the matter clearer—that of a gas expanding and doing work.

Let us consider first the case of a perfect gas expanding adiabatically and doing maximum external work. The relation between pressure and volume is $pv^\gamma = p_0v_0^\gamma$, where p_0, v_0 , are the initial values of p, v . The temperature of the gas falls, and the maximum work in expanding from v_0 to v is easily shown to be

$$W = \frac{p_0v_0}{\gamma - 1} \left[1 - \left(\frac{v_0}{v} \right)^{\gamma-1} \right].$$

Now if the gas had been allowed to expand without doing work, its temperature would not have fallen, and the relation between pressure and volume would have been $pv = p_0v_0$. Thus if —on the analogy of the tension-length curve of muscle as usually determined— we were to construct a pressure-volume curve by allowing the gas to expand *without doing work*, the relation we should find for our diagram would obey the equation $pv = p_0v_0$, and the “ theoretical maximum work ” W' , in expanding to volume v , would be

$$W' = p_0v_0 \log_e \frac{v}{v_0}.$$

This latter quantity may be very considerably greater than W , the actual maximum work, owing to the fact that in our method of determining W'

we have artificially maintained too high a pressure by allowing no work to be done. If $v/v_0 = 10$, assuming $\gamma = 1.67$ as for a perfect gas, the ratio W/W' is 0.51 ; if $v/v_0 = 100$, then W/W' is only 0.31 . We see, therefore, how serious an error may arise if we assume, in this particular case, that the area of the pressure-volume curve obtained by expansion to various volumes without doing external work really represents the theoretical maximum work that the system can perform. Under no conditions, *without the provision of extra energy from outside*, can work be done equal to the area of this pressure-volume curve.

Let us now, instead of the case of a gas, consider that of the system, whatever it be, which provides the mechanical work of muscle. Instead of a rise of temperature, which determines the increase of pressure in the gas by which work may be done, we must think of a state of activity in the muscle, a change in the "intensity" of some physico-chemical factor through which the increase of tension is evoked. Such a change in "intensity" might be a rise in hydrogen ion concentration locally produced and causing an alteration in the colloidal or electrical condition of the contractile elements. We know that in relaxation this change is abolished "irreversibly," in the thermodynamic sense; the fact that work performed diminishes by an equivalent amount the heat produced in relaxation suggests that the process which occurs "irreversibly" in relaxation may occur "reversibly" at an earlier stage, with the performance of work. Thus the change of "intensity" which we regard as responsible for the rise of tension in the muscle may be abolished, either by relaxation or by the performance of work. In the case of the gas, heat communicated suddenly to the system and producing a rise of temperature can be employed in causing an expansion against a resistance; if not so used, however, it will finally be dissipated irreversibly by conduction and radiation. If the expansion of the gas be a free one, no work being done, the temperature will not fall, and the same amount of energy will have finally to be dissipated; if the expansion be one in which the work done is less than the theoretical maximum, the amount of energy finally dissipated as heat will be diminished only by an amount equivalent to the actual work performed. The behaviour of a muscle in a twitch is parallel to that of the gas expanding adiabatically, substituting for the temperature of the gas the unknown "intensity" factor which causes the physico-chemical change manifesting itself in the mechanical response.

Hitherto we have considered a physical process analogous to that of a single twitch, where there is no further supply of energy available during the expansion, and the change in the intensity factor produced suddenly at the start is alone

responsible for all that follows. In the contraction evoked by a tetanus the conditions are different; here a further supply of energy is available, so long as the stimulus is continued, and the analogous case is that of a gas maintained in contact with a reservoir of heat at constant temperature. If the expansion be slow and "reversible" the work done will be equal to that calculated from the pressure-volume diagram obtained in a manner similar to that employed for the tension-length curve of the muscle. If it be not so slow, heat may not be able to pass rapidly enough from the reservoir into the expanding gas, some extra energy will indeed be taken in, but not sufficient to account for the whole of the work done.

In the case of the muscle, a very slow shortening during a prolonged stimulus allows the intensity factor to be maintained continuously at its maximum level. In the more rapid shortening, however, which is necessary—if much work is to be done before relaxation sets in—during the contraction evoked by a short stimulus, the conditions are not "reversible," and the rapid performance of work diminishes the "intensity" factor, which is responsible for the contraction, more quickly than it can be restored to its full level by further activity due to continued stimulation. Consequently the tension exerted falls below the level of the tension-length curve, and must so fall even neglecting the effect of these irreversible factors which are included under the term "muscle viscosity."

We arrive therefore at the following conception of muscle activity in relation to the performance of work. There is some "intensity" factor I , at present unknown, possibly however a local change in acidity or a difference of electrical potential, the sudden increase in which, as the result of stimulation, is responsible for the mechanical response; this alteration in the intensity factor I is analogous to the rise of temperature of a gas, which, causing an increase of pressure, permits the performance of work. If, however, the energy so set free be not employed immediately in the performance of work it tends to be dissipated irreversibly in a process which is known as relaxation, and I returns to its resting level. During a prolonged stimulus we must regard this irreversible process of "relaxation" as occurring continuously side by side with further "contraction." Consequently, to maintain during a tetanus an active state of readiness to do work, energy must be provided practically at a constant rate: the speed of lactic acid formation is adjusted automatically to secure a certain constant intensity I of our unknown factor. This state of affairs is analogous to that existing in a gas the temperature of which is increased by contact with a reservoir of heat. Owing to conduction and radiation,

there must be a continuous supply of heat to the gas if its pressure is to be kept up. If the source of heat be withdrawn the temperature and pressure of the gas return, by irreversible changes, to their previous level.

In a muscle twitch where no further source of energy is available the performance of work diminishes the intensity I , just as in a gas the expansion against a load diminishes the temperature. The greater the amount of work accomplished, the less, in either case, will be the amount of energy to be dissipated in the final irreversible process, of relaxation in the one case, of fall of pressure in the other. In a more prolonged contraction, where the stimulus persists and energy continues to be supplied during the performance of work, the intensity I tends to be maintained at its maximal level by the provision of further energy; in the case of the gas, contact with the reservoir allows the temperature to be kept up in spite of work performed.

The analogy of a gas has been taken for the purpose of illustrating the present hypothesis, partly because the expansion of a gas is the familiar process in thermodynamics, partly to avoid the impression that we are advancing any specific theory of muscular contraction. The analogy of a gas, however, is imperfect, since the muscle is not a heat-engine but a chemical machine working at constant temperature. A suggestive analogy is that of an electric battery, producing a charge on the plates of a condenser, which then attract one another and, if released, can come together doing external mechanical work. This system also has different mechanical properties, according as we suppose the working plates to remain connected with, or to be cut off from, the battery, which supplies them with energy.

Let us take the case of a parallel-plate condenser and consider the work done when the plates approach one another from distance x_0 to distance x , under two sets of conditions :—

- (i) When the plates are initially charged, and the battery is then withdrawn before the plates move ;
- (ii) When the plates are kept charged to the full potential of the battery throughout the process of moving.

Case (i) is analogous to that of a single twitch, case (ii) to that of a slow maintained contraction. In case (ii), as the plates come closer together, the capacity of the condenser increases and current flows from the battery to keep up the potential; in case (i) however, where the battery is cut off after charging the plates, no further supply of current is available and the potential difference falls as the plates approach one another. The electric force between the

plates is greater in case (ii) than in case (i), as soon as the plates have begun to move. It can be shown that the work done in case (ii) is greater than in case (i) in the ratio x_0/x , and corresponding to the greater work more energy is taken from the battery. Hence, if connection with the battery be maintained throughout the process, more work will be done, and more energy used, than if it be cut off as soon as the plates are charged. Thus, an electro-chemical system can be made to show the same properties as muscle, the intensity factor I being represented by a potential difference. It would be easy to amplify the analogy, *e.g.*, by introducing a leak across the condenser to represent relaxation, but the matter is probably already sufficiently clear. It is obvious that our unknown intensity factor I need not necessarily be a temperature: it may be an electromotive force, as the above example shows, or a concentration, or any other factor possessing analogous physical properties.

According to Fenn any factor which increases the work done increases also the total energy set free. Variation of the distance through which his muscle was permitted to shorten, and variation of the load or of the setting of his inertia lever, causing an alteration of the work done, all resulted in a corresponding change of the total energy liberated. This we have been able entirely to confirm. We have examined the following factors, employing the Levin-Wyman ergometer :-

(A) *Variation of Speed of Shortening.* - It was shown in a previous paper (9) that changing the speed of shortening alters the work done: we have found it to produce a corresponding alteration in the total energy, in any contraction except a twitch.

(B) *Variation of Degree of Shortening Allowed.* - If the extent of shortening allowed be greater, the work is greater, and we have found, except in a twitch, that the total energy is greater also.

(C) *Variation of Moment of Release.* - It was shown in the previous paper (9) that a slight delay in releasing the ergometer produces a small but measurable increase in the work: there is a corresponding increase in the total energy. Later release diminishes the work; the total energy is diminished also. This factor will be further considered separately, in relation to the question of work done during relaxation.

Many experiments have been performed leading to the same results; typical examples of the effects found are shown in Table VI.

Table VI.

*Variation of Total Energy with Work.**A. Variation of Speed of Shortening.**Experiment of 16.5.27, sartorii at 0° C.—*

Piston adjustment (speed)	0	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{3}{4}$	1	$1\frac{1}{2}$	2
$\frac{1}{4}$ second, stimulus—							
Work	0	19 $\frac{1}{2}$	29 $\frac{1}{2}$	28	24	6	0
Heat	85	74	71 $\frac{1}{2}$	71	70 $\frac{1}{2}$	68	66 $\frac{1}{2}$
Total energy	85	95 $\frac{1}{2}$	101	99	94 $\frac{1}{2}$	74	66 $\frac{1}{2}$
$\frac{1}{2}$ second, stimulus—							
Work	0	28	36	—	26	5 $\frac{1}{2}$	0
Heat	105	97 $\frac{1}{2}$	94 $\frac{1}{2}$	—	91	80	75
Total energy	105	125 $\frac{1}{2}$	130 $\frac{1}{2}$	—	117	85 $\frac{1}{2}$	75

Experiment of 7.6.27, sartorii at 0° C. 1 second, tetanus—

Piston adjustment (speed)	0	$\frac{1}{4}$	$\frac{1}{2}$	1	$1\frac{1}{2}$
Work	0	24	44	30	5
Heat	99	105	120	118	94
Total energy	99	129	164	148	99

Experiment of 21.4.27, sartorii at 0° C.—

Piston adjustment (speed)	0	$\frac{1}{4}$	$\frac{1}{2}$	1
0.06 second, stimulus—				
Work	0	12 $\frac{1}{2}$	19	14
Heat	56	43 $\frac{1}{2}$	37 $\frac{1}{2}$	34 $\frac{1}{2}$
Total energy	56	56	56 $\frac{1}{2}$	48 $\frac{1}{2}$
0.25 second, stimulus—				
Work	0	21 $\frac{1}{2}$	28 $\frac{1}{2}$	22 $\frac{1}{2}$
Heat	77	65	60 $\frac{1}{2}$	60
Total energy	77	86 $\frac{1}{2}$	89	82 $\frac{1}{2}$
0.50 second, stimulus—				
Work	0	26 $\frac{1}{2}$	36	26 $\frac{1}{2}$
Heat	93	84	81	77
Total energy	93	110 $\frac{1}{2}$	117	103 $\frac{1}{2}$

Experiment of 10.3.27, sartorii at 13° C., 0.1 second, tetanus—

Piston adjustment (speed)	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$
Work	0	21	36	42	45	36
Heat	137	126	126	124	127	125
Total energy	137	147	162	166	172	161

Experiment of 15.6.27, sartorii at 14 $\frac{1}{2}$ ° C.—

Piston adjustment (speed)	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	3
0.03 second, stimulus—						
Work	0	11 $\frac{1}{2}$	16	19	18	14
Heat	72	66 $\frac{1}{2}$	62	60	59 $\frac{1}{2}$	59
Total energy	72	78	78	79	77 $\frac{1}{2}$	73
0.10 second, stimulus—						
Work	0	21	31	40	36	31
Heat	116	112	111	110	112	117
Total energy	116	133	142	150	148	148
0.25 second, stimulus—						
Work	0	38	59	67	63	—
Heat	178	178	174	180	187	—
Total energy	178	216	233	247	250	—

Table VI (continued).

Experiment of 9.6.27, sartorii at 15° C.—

Piston adjustment (speed)	0	$\frac{1}{4}$	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	3	4
0.10 second—								
Work	0	7	$14\frac{1}{2}$	23	29	$28\frac{1}{2}$	24	$21\frac{1}{2}$
Heat	94	91	89	89	91	$92\frac{1}{2}$	99	100
Total energy	94	98	$103\frac{1}{2}$	112	120	121	123	$121\frac{1}{2}$
0.25 second—								
Work	0	—	$25\frac{1}{2}$	41	$50\frac{1}{2}$	$48\frac{1}{2}$	31	—
Heat	171	—	169	176	174	178	168	—
Total energy	171	—	$194\frac{1}{2}$	217	$224\frac{1}{2}$	226	199	—

B. Variation of Degree of Shortening Allowed.*Experiment of 16.3.27, sartorii at 15° C., 0.2 second, tetanus—*

Shortening allowed, mm.	0	1.7	3.3	5	7
Work	0	16	31	48	$63\frac{1}{2}$
Heat	182	186	181	177	167
Total energy	182	202	212	225	$230\frac{1}{2}$

Experiment of 22.3.27, sartorii at 15° C., 0.3 second, tetanus—

Shortening allowed, mm.	0	3.0	5.1	7.6	9.7
Work	0	18	31	37	50
Heat	125	143	148	143	132
Total energy	125	161	179	180	182

Experiment of 8.3.27, sartorii at 13° C., 0.1 second, tetanus. Three separate series, each with a "reverse," averaged—

Shortening allowed, mm.	0	1	2	3	4
Work	0	14	27	$39\frac{1}{2}$	$45\frac{1}{2}$
Heat	107	113	113	106	102
Total energy	107	127	140	$145\frac{1}{2}$	$147\frac{1}{2}$

In considering the variation of work and energy with moment of release we are necessarily involved with the question, originally raised by Fenn, of work done during the process of relaxation. According to Fenn (2) such work leads to a decrease in total energy set free. According to Azuma (10) shortening during relaxation has this effect, whether work be done or not. We have been unable to confirm this effect, as the experiments of Table VII show. There observations made on muscles released at, or after, the moment when the stimulus ended are given in italics, and a glance at them shows that, although a considerable amount of work may be done, the total energy is not less, by a significant amount, than that for the isometric contraction. An early release, during the stimulus, causes an extra liberation of energy over and above the isometric level; a release at, or shortly after, the end of the stimulus has the same, but a smaller, effect; a release appreciably after the end of the stimulus, but sufficiently early to allow a considerable amount of work still to be performed, leaves the total energy at the isometric level. We are unable to reconcile this conclusion with the results of Fenn and of Azuma, or with those

which we obtained ourselves before by a different method (see Fenn (2), p. 391). We can only suppose that some technical disturbance in the muscle or the instruments affected the previous results, for we are inclined to put more trust in the present experiments with the Levin-Wyman ergometer, than in those made with the earlier devices for recording, or allowing for, the external work performed.

The Levin-Wyman ergometer works very sweetly if properly constructed and adjusted, without vibrations, jerks, or discontinuities of any kind. See fig. 1. In this respect it is far superior to the mechanical devices previously employed. In one of these, for example, the lever hit a stop at the end of its course, whereby presumably a certain amount of vibration was communicated along the thread to the muscle. Such jerks and vibration may well have affected the contact of the muscle with the thermopile, and therefore the deflections obtained. In the case of such small readings as are given by single shocks, and of such small differences as occur with muscles released during relaxation, these technical disturbances may have led to appreciable error. We have not attempted to repeat the experiments with the older mechanical arrangements: the ergometer is so much more satisfactory an instrument that, having worked with both, we have no hesitation in preferring results obtained with it.

In the present experiments the muscle was automatically brought back to its original "long" position, immediately it relaxed, by the small initial tension (2 or 3 grams). This was not done in all the previous experiments, and so a small error may have been caused.

Table VII.

Variation of Work and Total Energy with Moment of Release.

(Observations referring to releases at or after the end of the stimulus are given in italics.)

Experiment of 11.3.27, at 13° C., 0.1 second, tetanus—

Moment of release.

seconds	0	0.015	0.030	0.045	0.061	0.091	0.121	0.167	0.212	∞
Work	40	40	43	39	38	33	26	15	5½	0
Total energy . . .	152	154	155	152	150	147	138	127	122½	124

Experiment of 28.3.27, at 14° C.—

Release, seconds . . . 0.015 0.030 0.045 0.061 0.076 0.091 0.106 0.137 ∞

0.03 second, stimulus—

Work	21	19	16	14	—	7	—	—	0
Energy	99	91	85½	84½	—	83	—	—	87

0.09 second, stimulus—

Work	—	—	—	—	20	—	16	9	0
Energy	—	—	—	—	146	—	134	128	136

Experiment of 31.3.27, at 5° C. Two separate series, averaged, at 0.15 second, stimulus; one series at 0.3 second, stimulus—

Release, seconds . . . 0.121 0.151 0.197 0.272 0.303 0.348 0.379 0.424 0.531 ∞

0.15 second, stimulus—

Work	22½	21	18	13	—	—	5	—	—	0
Energy	82	79	74½	70	—	—	67½	—	—	69

0.30 second, stimulus—

Work	—	—	—	—	22	18	—	15	7½	0
Energy	—	—	—	—	103	97½	—	94½	93½	92

Table VII (continued).

Experiment of 1.4.27, at 5° C., 0.2 second, tetanus—

Release, seconds	0.197	0.242	0.303	0.363	0.425	∞
Work	22½	18½	15	11½	6½	0
Total energy	94½	89½	85	82½	82½	82½

Experiment of 12.5.27, at 0° C., 0.5 second, tetanus—

Release, seconds	0.024	0.127	0.285	0.50	0.68	∞
Work	38	39	36	29	22	0
Total energy	154	153	148	133	123	110

Experiment of 13.5.27, at 0° C., 0.5 second, tetanus—

Release, seconds	0.024	0.127	0.285	0.50	0.68	∞
Work	44½	49	44	35	24½	0
Energy	149½	154	146	132	118	118

Experiment of 14.5.27, at 6° C.—

Release, seconds	0.024	0.119	0.25	0.365	0.50	0.68	∞
0.25 second, stimulus—							
Work	29½	29	20	13	7½	—	0
Energy	133½	127½	113	102½	100½	—	103
0.5 second, stimulus—							
Work	44	—	34	—	20	10	0
Energy	198	—	177	—	149	136	138

Experiment of 21.5.27, at 15° C., 0.1 second, tetanus—

Release, seconds	0	0.032	0.10	0.174	∞
Work	44½	44	31	17	0
Energy	150½	145	123	110	110

The conclusion from Table VII that work performed some time after the stimulus is over has no effect on the total energy liberated, is entirely in keeping with the hypothesis we have advanced above. The reservoir of energy maintaining the intensity *I* in the contractile regions is presumably withdrawn when the stimulus ends, so that the contrary effect previously stated, viz., an absorption of energy owing to work in relaxation, would be extremely difficult to account for. Apparently work affects the total energy only if performed during, or shortly after, the stimulus, i.e., before the channel of energy supply required to maintain the intensity *I* is closed.*

The absence of an effect of work done during relaxation on the total energy

* Work done at any time in the twitch evoked by a single shock appears to have no effect on the total energy; in a tetanus, as shown in Table VII, work done shortly after the end of the stimulus may still lead to a positive excess energy. It is only when work is done appreciably after the end of the stimulus that it has no effect on the total energy. Apparently in a twitch the channel of energy supply closes immediately after the shock, while in a tetanus it remains open for an appreciable time. This conclusion is borne out by the observation recorded in our paper (12) on the isometric twitch, that the mechanical response to the later elements in a tetanic stimulus is considerably delayed—see particularly fig. 1, p. 391, in that paper.

set free was so crucial, from the point of view of the hypothesis advanced here, and we were so loath, without the fullest evidence, to contradict previous work on the subject, that we undertook a new series of observations as described at the beginning of this paper. In Table VIII the excess energy in the working case is given, as a fraction of the isometric heat, for a number of experiments in which the muscle was released only after the stimulus had ended. To show that a considerable amount of work was done in these cases, in spite of the late release, the mechanical efficiency also is given. The average value of the mechanical efficiency in these experiments, for an early release, was about 23 per cent., and the numbers given for the cases of later release show that a considerable fraction of the maximum work was still being accomplished.

Table VIII.

Effect of Work Done after the Stimulus is over on the Total Energy set free.

Dates are all 1928. Stimulus duration in seconds. Release in seconds after the *beginning* of stimulus. Mechanical efficiency as per cent. Excess energy, working, as fraction of isometric heat. S denotes silver-frame thermopile.

Date	16.7.	16.7.	18.7.	20.7.	20.7.	21.7.	21.7.	21.7.
Thermopile	S	S	S	S	S	S	S	S
Temperature, ° C.	18	18	18	18	18	18	18	18
Stimulus duration	0.03	0.10	0.10	0.08	0.08	0.04	0.08	0.08
Release at	0.06	0.13	0.13	0.13	0.14	0.13	0.11	0.14
Efficiency	21	17½	15	14	10	4	15	6
Excess energy	0.01	-0.01	+0.07	-0.02	-0.01	-0.05	-0.01	+0.02
Date	24.7.	11.7.	11.7.	11.7.	13.7.	13.7.	25.7.	26.7
Thermopile	S	S	S	S	S	S	S	S
Temperature, ° C.	18	0	0	0	0	0	0	0
Stimulus duration	0.08	0.10	0.10	0.10	0.10	0.40	0.40	0.40
Release at	0.14	0.19	0.29	0.38	0.28	0.63	0.49	0.59
Efficiency	7	22	16½	11½	18½	15½	18½	20½
Excess energy	-0.02	+0.04	-0.01	-0.02	+0.09	+0.08	+0.10	+0.07
Date	17.8.	1.8.	8.8.	14.8	22.8.	24.8.		
Thermopile	S	—	—	—	—	—		
Temperature, ° C.	0	0	0	0	0	0		
Stimulus duration	0.30	0.30	0.30	0.30	0.3	0.3		
Release at	0.59	0.59	0.59	0.59	0.49	0.49		
Efficiency	16	15	11½	10	12	15		
Excess energy	+0.04	-0.01	-0.05	-0.03	-0.04	-0.01		

A survey of the figures in Table VIII leaves little doubt that the performance of work appreciably after the stimulus ends has no effect upon the total energy. The average* excess energy in the experiments at 18° C. is practically zero :

* See note p. 4 above. In several experiments in which both twitch and tetanus were used it was observed that the excess energy (over isometric) when doing work (a) with an early release in a twitch and (b) during relaxation only in a tetanus, were small

in the experiments at 0° C. it is about +0.02 of the isometric heat. For a release at the commencement of the stimulus it would be about +0.30. Thus during relaxation, as in the single twitch, the performance of work as such has no effect on the energy set free; variation of the total energy can be produced only by allowing the muscle to do work while it is being stimulated, or during a short interval immediately following the stimulus.

Discussion.

In this way, apparently, the new facts and hypotheses can be reconciled with the old. The tension-length diagram represents the maximum work only in the case of contractions so slow that the "intensity" I can be fully maintained by the continuance of the stimulus; just as in the gas the pressure-volume diagram derived from the equation $p^n = \text{constant}$ represents the maximum work only if the expansion be so slow that heat can be absorbed from the reservoir in amount sufficient to compensate for the work done. It does not, however, follow that the muscle is not to be treated as an elastic body: such a conclusion applied to the gas would obviously be absurd. A rapid shortening of the muscle is like a rapid adiabatic expansion of the gas—the source of external energy is no longer available, and work done causes a decrease in the "intensity" (temperature in gas, or our unknown factor I in muscle) by which the work was rendered possible. The active muscle, like the heated gas, is still an elastic body, but its true tension-length relation depends upon the conditions of its shortening, whether "adiabatic" on the one hand, or "isothermal" on the other, or—in practice—somewhere between the two.

It is clear that if this hypothesis be correct it must have some bearing upon the phenomena which are described as the "viscous-elastic properties" of muscle. If a muscle, during a continued stimulus, shorten rapidly, it will do less work, partly because the work done lowers the intensity I too rapidly for the latter to be maintained at its maximal level; because—to use the analogy of the gas—the process has occurred "adiabatically" instead of "isothermally." This, as well as irreversible viscous loss, is the reason why work done diminishes with speed of shortening. That there remains, however, a real *but of the same sign*, sometimes both positive, sometimes both negative. Any technical error, such as might arise if the face of the thermopile were not plane, which affected the isometric and the working heat differently, would produce such an effect; it seems unlikely therefore that the first excess (a) is really positive and the second (b) negative; both are probably zero, and the variations shown in Tables I and VIII due to a technical error of the kind suggested.

viscous-elastic effect is readily seen in the case of the single twitch, where no further supply of energy is in any case available, and yet the work done diminishes to a negligible amount as the speed of shortening is made more and more rapid. The facts of the "viscous-elastic" properties of muscle are not affected by the results and hypothesis of the present paper; in seeking, however, for their explanation we must bear in mind that although irreversible effects analogous to viscosity do undoubtedly play an important rôle, the Fenn effect and the diminution with work done of the intensity factor I , are probably of equal importance.

We have not considered so far the phenomena shown by a muscle being stretched, instead of allowed to shorten. Fenn (2) showed that stretching a muscle during its contraction causes a decrease in the total energy set free, the converse of allowing it to shorten. This in itself suggested a thermodynamical basis for the relation between work and total energy. The effect was confirmed by Wyman (7) and is of considerable magnitude. There is no difficulty in reconciling it with—indeed, it is a necessary consequence of—our hypothesis. Taking the case of the gas, a compression causing a rise of temperature would tend to prevent the system from absorbing heat from the reservoir. In the muscle, if shortening, with the performance of work, tends to cause reversibly the same process as occurs irreversibly in relaxation, viz., the lowering of the "intensity" I , then lengthening with the absorption of work must tend to cause reversibly the opposite process, to hinder the irreversible change which is manifest in relaxation. What the intensity factor I may be and how it works cannot be stated at present; provided, however, that it bears the same relation to the structures of a muscle as temperature bears to a gas the converse phenomena in the case of stretching must necessarily occur.

We have assumed, in the above discussion, that all the work performed by the muscle is employed in overcoming an external resistance. We have taken no account of "viscous" resistance to shortening. The analogy of the gas may tell us perhaps how the matter should be regarded. Imagine that the expansion is so rapid that a considerable fraction of the mechanical energy theoretically available is wasted irreversibly in stirring and warming the gas itself. The ideal case, where no work is done in expansion, we have already discussed. It is clear that the energy absorbed from the reservoir to maintain the temperature of the gas during its expansion is equal to the actual external mechanical work performed, and if the latter be diminished by irreversible viscous loss in the gas, then the energy absorbed is diminished correspondingly.

Similarly if a muscle be allowed to shorten, doing no external work, the total energy liberated should be the same as in an isometric contraction. If the muscle does external work less in amount than its maximum, owing to rapid shortening against its own internal resistance, then one might expect to find extra energy corresponding only to the actual external work performed. We have made many sets of observations of the work performed and the total energy liberated at a series of increasing speeds—see *e.g.*, Table VI above. We have always found that at the higher speeds, where the work is less, the total energy is less also, showing that internal work against viscous resistance has little effect upon total energy set free.

The fact that the energy liberated in a twitch is independent of whether work be done or not supplies an important extension of the “all-or-none” theory of muscular activity. So long as it was believed that the energy varied in a twitch, as in a tetanus, with work done, *i.e.*, with events occurring *after* the stimulus, it was not possible to suppose that the unit of response of a muscle fibre depends only on conditions existing up to the time of the stimulus. The “all-or-none” view can now apparently be re-instated in this respect. If a muscle, under specified conditions existing prior to stimulation, be excited by a single shock the response cannot be varied in magnitude by any mechanical factors affecting the manner in which the twitch is carried out. The all-or-none theory is not applicable, in any case, to a tetanus, so the existence there of the Fenn effect provides no objection to it.

The heart-beat is usually regarded as analogous to the single twitch of a skeletal muscle. Accepting this view we should expect the energy liberated by the heart to be independent of the conditions existing subsequently to the initiation of its beat. It should depend, as Starling always maintained, upon the mechanical conditions obtaining at the extreme end of diastole, *viz.*, upon the length of the fibres, but not upon the resistance encountered during its contraction. In a recent paper (13), Clark and White have shown that the oxygen consumption of the frog's heart varies with the diastolic volume, but is not influenced by changes in the resistance against which the heart contracts. The energy liberated in a heart-beat depends, therefore, only on the initial conditions, as it does in a muscle twitch.

Summary.

1. In the tetanic contraction of a frog's muscle, as found by Fenn, more energy is liberated if work be performed. The excess energy is relatively

greater for greater durations of stimulus, becoming equal to, or even greater than, the work in the more prolonged contractions.

2. In a single twitch, however, the total energy set free is the same whether work be done or not. The work done is simply subtracted from the heat liberated in relaxation.

3. In a tetanic contraction, as in a twitch, work done diminishes relaxation heat by an equivalent amount. Excess energy, due to work, appears as an increment in contraction heat.

4. These facts support the view that work and relaxation heat are derived from the same source, viz., the mechanical potential energy of the active muscle.

5. Relaxation heat, in twitch or tetanus, is equivalent only to about one-half of the area of the tension-length curve. It is difficult, therefore, to suppose that the latter represents the mechanical potential energy of the active muscle. It would seem that the potential energy is better measured by the relaxation heat.

6. Work done by a muscle in the phase of relaxation, *i.e.*, appreciably after the stimulus is over, has no effect upon the total energy set free.

7. In confirmation of Fenn, any factor which increases, or decreases, the work done during the contraction phase of a tetanic response, similarly increases, or decreases, the total energy liberated.

8. The simplest statement of the phenomena is that an active muscle, performing mechanical work, sets free an excess of energy only if it be allowed to shorten and do work during the stimulus or a short period thereafter.

9. The fact that the energy liberated in a twitch is independent of whether work be done or not provides an extension of the "all-or-none" theory of muscular activity. The magnitude of the unit response of a muscle (the twitch) is determined only by the conditions existing prior to, and at the moment of, the shock which evoked it, and not by any factors affecting it subsequently.

10. If the heart-beat be regarded as a single twitch these results confirm Starling's view that the energy liberated by a heart depends only on its initial filling, and not upon the resistance which it encounters during the course of its contraction.

11. The necessity is indicated of a new thermodynamic outlook on the activity of muscle. Contraction is due to the increase of some unknown "intensity" factor, analogous to the temperature of a gas, or the potential difference between the plates of a condenser. This factor is diminished by the performance of

work. It can be maintained at its full value, during the performance of work, only by continued stimulation, *i.e.*, by the further liberation of energy.

12. On this view the tension-length curve does not represent the theoretical maximum work, except for the case of a muscle maintained in contraction by a tetanus, and shortening slowly.

13. The nature of the viscous-elastic properties of muscle is discussed in view of these new facts and hypotheses.

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The Mechanism of Secretion in the Thyroid Gland.

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[PLATE 1.]

PART I.—THE NORMAL GLAND IN PROLONGED FUNCTIONAL ACTIVITY.

The thyroid gland presents a peculiar problem in its mechanism of secretion. In previous publications* we have shown that during rest the colloid is secreted into the centre of an alveolus—an enclosed space in which it accumulates. When the gland is stimulated to activity by exposure to cold, or in sympathetic fever, there is a disappearance of the colloid from this enclosed space, together with an opening up of the inter-alveolar and intra-alveolar capillaries. What happens now when the gland continues its activity after most of the colloid has disappeared from the alveolar lumen? Do the thyroid cells continue to pass their specific hormone into the alveolar lumen, from which it is then poured into the circulation? Or, do they reverse the direction and pass the specific hormone directly into the circulation? In the second part of this paper we shall show that such a reversal occurs under pathological conditions, so that it is a matter of importance to determine whether it takes place also under physiological conditions.

Information on this point can be obtained by a study of the Golgi apparatus in the thyroid cells. Recent cytological work has shown that the Golgi apparatus is intimately related to certain phases of the process of secretion. In gland cells the specific products of secretion accumulate in relationship with the Golgi apparatus, which in these cells is always situated at the side of the nucleus directed towards the secreting surface of the cell. At the onset of secretory activity the Golgi apparatus enlarges, spreading out away from the nucleus towards the lumen of the alveolus. Droplets of secretion make their appearance in contact with the surface of the Golgi apparatus, and, breaking away from it, pass out into the cytoplasm towards the lumen. The position of the Golgi apparatus—its polarity—gives, therefore, a clue as to the direction

* Cramer, W., 'Fever, Heat Regulation, Climate and the Thyroid Adrenal Apparatus,' London, 1928 (Longmans, Green & Co.).

of the process of secretion. In a previous paper we have shown that the Golgi apparatus of the thyroid cells is always situated between the nucleus and the alveolar lumen, that it undergoes the changes just described when the gland is stimulated to activity, and that no reversal of polarity can be obtained under the conditions which we studied.*

Cowdry† has recorded an observation on the thyroid gland of a guinea pig in which a few cells showed a reversal of the polarity of the Golgi apparatus. The state of functional activity of the gland in which it occurred was not known, however, and the appearances figured by him do not show the Golgi apparatus in a state of activity. Such exceptional occurrences of reversal of a resting Golgi apparatus have also been observed occasionally in the cells of other glands.‡ Their significance is unknown. It has been attributed to mechanical displacement.

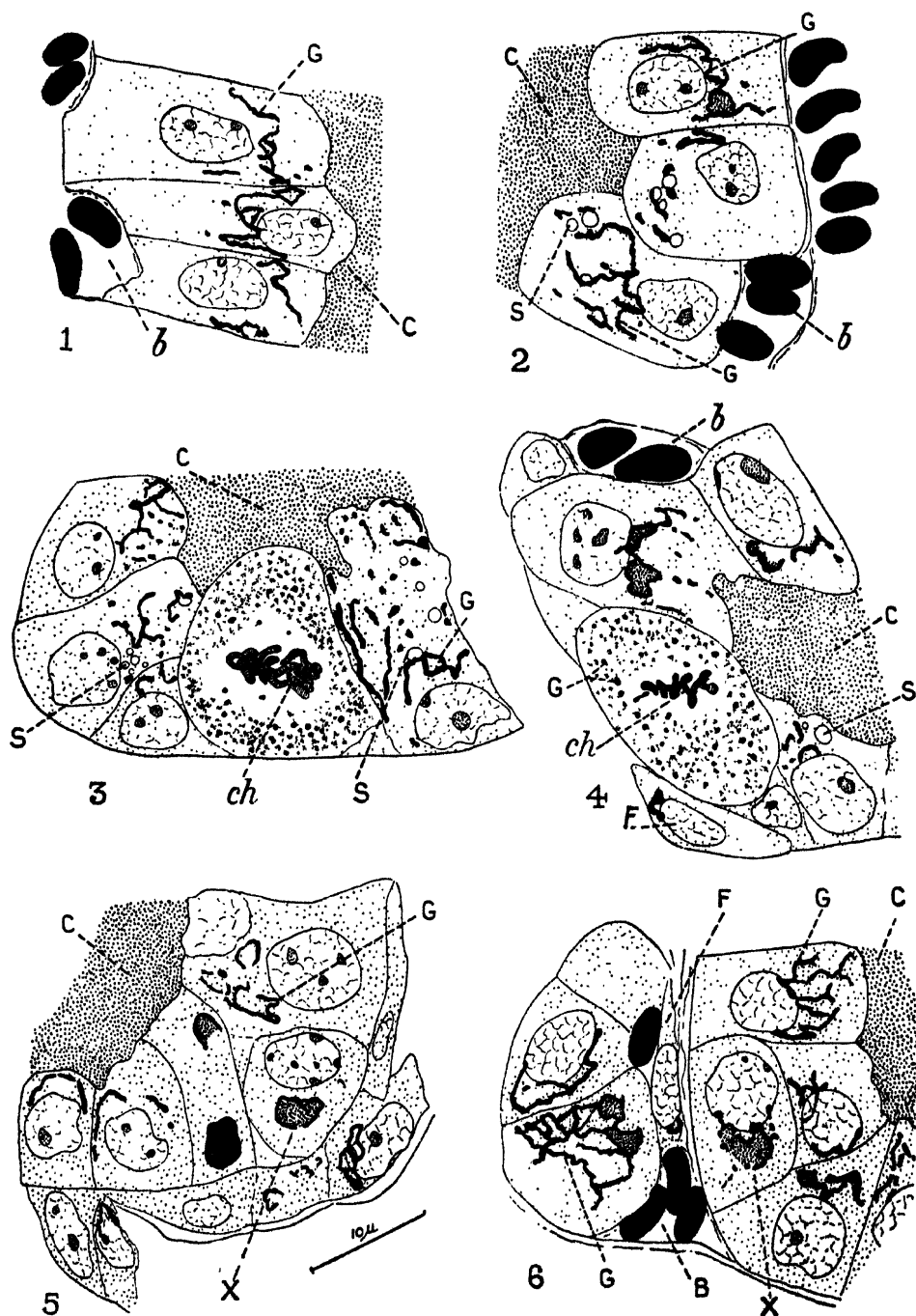
As stated above, the peculiar mechanism of secretion in the thyroid gland is such that a thyroid cell might conceivably be possessed of a double polarity. The lack of evidence in favour of such a view in our previous observations might have been due to insufficient stimulation of the gland. We have therefore carried out a further series of experiments in which we endeavoured to obtain the maximum degree of prolonged activity. The observations were made on rats which were exposed to cold by being kept in an ice chest for varying periods of time. In some animals the effect was intensified by epilating them.

The most striking results were obtained in the thyroid glands of rats which were kept at room temperature, and then epilated and exposed to the cold for 24 hours. The glands of such animals show the intense congestion of both inter- and intra-alveolar capillaries to which one of us has previously drawn attention. Hæmorrhage into the centre of alveoli is of frequent occurrence. The form of the Golgi apparatus varies considerably in the glandular cells. Most of the cells show the condition of extreme secretory activity. The Golgi apparatus is spread out in the cytoplasm, and droplets of secretion are discernible in contact with it. There is considerable variation both in the form and arrangement of the apparatus. In the great majority of the cells it is situated between the nucleus and the lumen of the vesicle, but a few cells are to be seen in which it has been displaced. Such cases give one the impression

* Cramer and Ludford, 'J. of Physiology,' vol. 61, p. 398 (1926).

† Cowdry, 'General Cytology,' Chicago, p. 336 (1924).

‡ Bowen, 'Q.J.M.S.,' vol. 10, p. 395 (1926).



FIGS. 1-6.—Groups of cells from the vesicles of thyroid glands of rats which have been epilated and exposed to cold for 24 hours.

1.—Two gland cells with normally situated Golgi apparatus (G.); middle cell with apparatus reversed in position.

FIG. 2.—Upper cell showing reversal in polarity of Golgi apparatus (*G.*). Two lower cells with secretion droplets formed in contact with the surface of the Golgi apparatus.

FIG. 3.—Group of thyroid cells in stage of active secretion.

FIG. 4.—Cells at early stage of secretory activity.

FIGS. 5 and 6.—Groups of thyroid cells showing normal and abnormal positions of the Golgi apparatus.

B., inter-alveolar capillary; *b.*, intra-alveolar capillary; *C.* colloid; *Ch.*, chromosomes; *F.*, fibroblast; *G.*, Golgi apparatus; *X*, cells wedged in at the base of gland cells.

All the figures are drawn from sections of material prepared by the modified Kopsch method previously described.*

that there has been a mechanical displacement of the Golgi apparatus rather than a reversal of polarity.

Figs. 1 and 2 show cases of a definite alteration in position of the apparatus taken from different alveoli. In fig. 1 no secretion droplets are visible in any of the cells, but in two of the cells depicted in fig. 2 there are numerous droplets (*S.*). They are, however, only present in the two cells with a normally situated Golgi apparatus. This state of affairs is typical of all the cells exhibiting reversal of polarity of the apparatus which we have observed. We have been unable to find any cells, with a reversal in polarity of the Golgi apparatus, containing secretion droplets which appeared to be passing directly into a capillary.

The typical form of secretory activity is represented in figs. 3 and 4, which show groups of gland cells amongst each of which a cell at the metaphase of mitosis is seen (*ch.*, chromosomes). The occurrence of mitoses may in itself be regarded as indicating increased functional activity. The two upper cells in fig. 4 are typical of the early stages of secretory activity. The secreting cells of fig. 3 are characteristic of the advanced stage of the secretion process. We find, therefore, in confirmation of our previous observations, that stimulation of the normal thyroid of an animal causes the Golgi apparatus of the cells to enlarge, that secretion droplets make their appearance in contact with its surface and, on attaining a certain size, they pass into the cytoplasm of the cell, and are ultimately secreted into the colloid of the vesicle. We conclude, therefore, that continued functional activity of the normal gland does not alter the direction in which the cells secrete.

We have frequently observed in sections of the thyroid glands of animals exposed to cold, cells lying at the base of, or wedged in between, the typically arranged glandular cells. Two such cases are represented in figs. 5 and 6 (*X*).

* Ludford, 'Jour. R.M.S.' p. 107 (1925).

The Golgi apparatus (*G.*) of these cells (**X**) occupies an abnormal position. It is neither directed towards the lumen of a vesicle or towards a capillary. It is of interest to note that these cells do not contain secretion droplets. Their function may be to replace exhausted gland cells.

Summary.

The cells of the normal thyroid gland discharge their secretion into the lumen of a vesicle, from whence it passes into the blood stream. Even during prolonged increased functional activity there is no alteration in the direction of the discharge of secretion. There is no evidence that in the normal gland the cells secrete directly into the blood capillaries.

PART II.—THE GLAND IN EXOPHTHALMIC GOITRE.

The study of the mechanism of secretion in the normal thyroid gland, reported in the first part of this paper, has led to the conclusion that in prolonged activity of the organ the gland cells continue to secrete toward the alveolar lumen so that the specific hormone first collects there, and then passes into the blood stream. It seemed of interest to compare these conditions with the mechanism of secretion in abnormally active glands, that is to say, in exophthalmic goitre. As all our experimental work on the normal gland had been carried out with rats and mice, we were particularly fortunate in obtaining a mouse in a condition closely resembling exophthalmic goitre as it occurs in man. In addition to a pronounced exophthalmos and tremor, there was a nodular enlargement of the thyroid gland, which microscopically had the typical appearance of the human thyroid gland in exophthalmic goitre (see fig. 2). There was also an enlargement of the cervical lymph glands, and of the adrenals. The appearance of the latter has been described and figured elsewhere.*

The mouse was placed at our disposal by Prof. Leonard Hill, F.R.S., to whom we are greatly indebted for this rare and valuable material. The condition had developed spontaneously in an animal belonging to his stock of mice. We have also examined human thyroid glands of three cases of exophthalmic goitre. The material was obtained from operations under local anaesthesia, and fixed immediately after the operative removal. For the human material we are obliged to Mr. T. P. Dunhill, F.R.C.S., of St. Bartholomew's Hospital, who most kindly gave us every facility to obtain the material under the most favourable conditions. All the three cases had been subjected before operation to treatment

* Cramer, W., 'Fever, Heat Regulation, Climate and the Thyroid Adrenal Apparatus,' London, 1928 (Longmans, Green & Co.).

with iodine, which is adopted by Mr. Dunhill as a routine method with the object of reducing the hyperactivity of the gland before its operative removal.

We are indebted to Dr. G. S. Sansom, of the Anatomy Department, University College, for the two photomicrographs in Plate 1. For the study of the cell structures, preparations were made by the Schridde method for mitochondria, and by the modified Kopsch method for the demonstration of the Golgi apparatus.*

Secretion in the Thyroid Cells in Exophthalmic Goitre of the Mouse.

A general view of the thyroid gland in the case of exophthalmic goitre of the mouse, which we have studied, is given in fig. 2, Plate 1. The gland showed a remarkable deviation from the normal structure. In section it is seen to be divided up into several lobules. Portions of three of them are seen in the photograph. Although there are indications of the normal vesicular structure, yet most of the cells are arranged in irregular groups separated by connective tissue, containing numerous congested blood vessels.

The gland cells vary considerably in size. A glance at the figs. 7 to 12 will give some indication of the extent of the variation. In the enlarged cells both the Golgi apparatus and mitochondria are increased in size. Unlike the condition in the normal gland, even under conditions of hyperactivity, the Golgi apparatus of the cells shows quite frequently a complete reversal of polarity. Fig. 7 shows a group of cells from a part of the gland where the vesicular structure is still retained. It will be observed that while in the lower cells the apparatus (*Gn.*) is occupying its normal position between the nucleus and the lumen of the vesicle, in the two upper cells the apparatus (*Gr.*) is completely reversed, and in both cells is directed towards a capillary. The same reversal of polarity is shown in hypertrophied cells in fig. 8. The middle cell of the three has the reversed Golgi apparatus directed towards a capillary, as in fig. 7.

In the first part of this paper we have pointed out that when the thyroid gland is stimulated to intense secretory activity a reversal in polarity of the Golgi apparatus can occasionally be seen. We have not been able to find any evidence of actual secretion formation in relationship with the Golgi apparatus in such cells. It seems, therefore, highly probable that the displacement of the Golgi apparatus, which occurs sometimes in the normal thyroid cells, may be due simply to mechanical derangement. This explanation does not apply to the reversal of polarity of the apparatus which occurs in exophthalmic

* Ludford, 'Jour. R.M.S.,' p. 107 (1925).

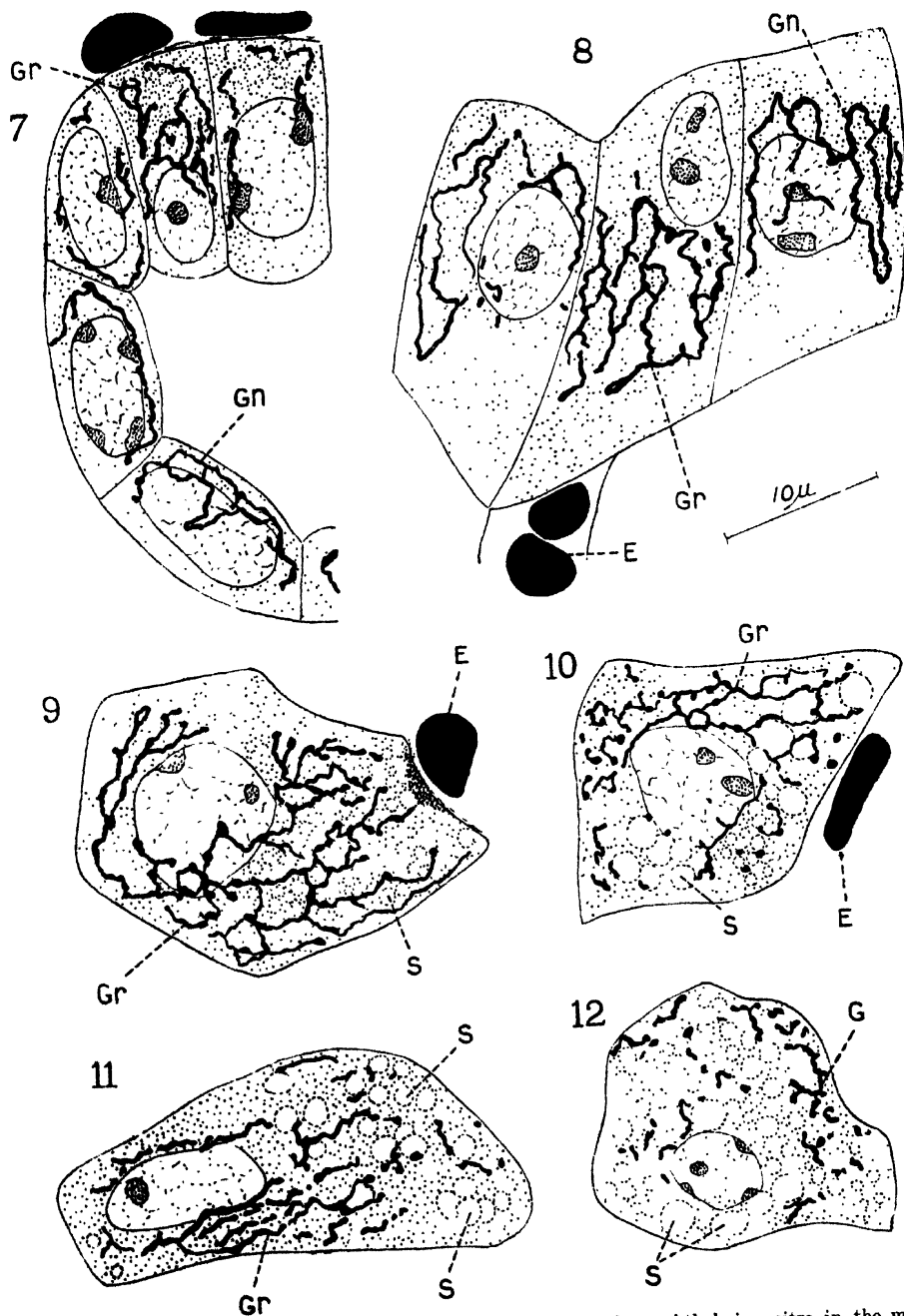
goitre. In this condition secretion droplets can be seen forming in relationship with the reversed Golgi apparatus, which exhibits the characteristic changes associated with secretory activity in gland cells. This process is shown in figs. 9 to 12. Fig. 9 is a cell with the enlarged apparatus (*Gr.*) characteristic of the onset of secretory activity. The apparatus (*Gr.*) it will be observed is directed towards a capillary (*E.*, erythrocyte). Fig. 10 shows secretion droplets (*S.*) forming in contact with the Golgi apparatus (*Gr.*). In the next figure (11) the secretion droplets (*S.*) are breaking away from the apparatus and passing towards the capillary. While, finally, in fig. 12 the apparatus (*G.*) is fragmented, numerous droplets of secretion (*S.*) are present in the cytoplasm and the nucleus presents the characteristic appearance of an exhausted cell.

In previous observations on the normal thyroid gland we have shown that an increase in cellular activity is accompanied by an increase in the mitochondrial-cytoplasmic interfaces as well as by enlargement of the Golgi apparatus.* The increase in number and enlargement of the mitochondria is very striking in the thyroid cells in exophthalmic goitre. Fig. 13 shows one of the thyroid cells with mitochondria (*M.*) in the form of granules and rodlets, while fig. 14 represents the stage of intense secretory activity with the mitochondria (*M.*) represented by long filaments. This condition, as we have shown previously, can be induced in the normal animal by stimulating the thyroid to intense activity either by exposure to cold, or by the injection of β -tetrahydronaphthalamine.

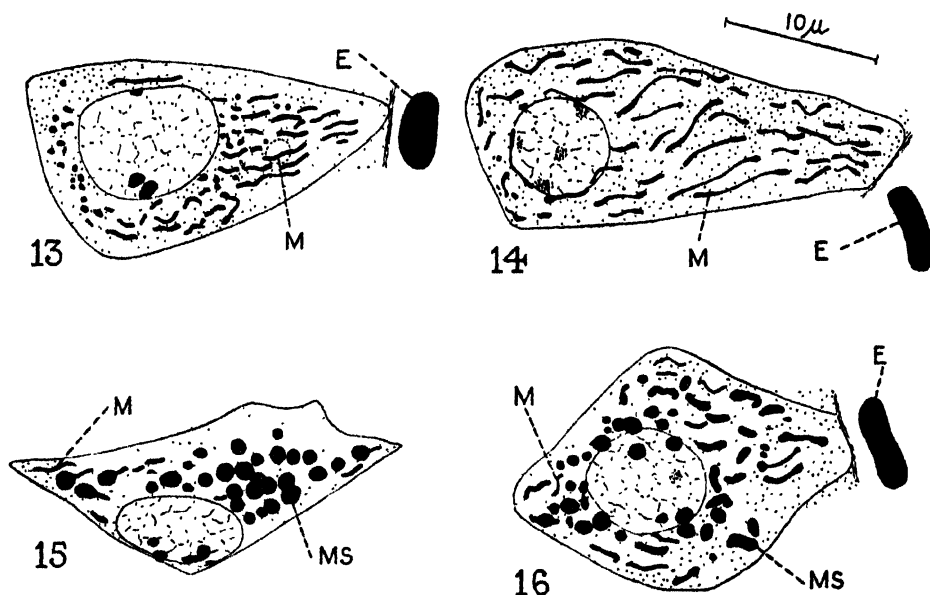
In certain areas of the exophthalmic thyroid the mitochondria are swollen up so as to form relatively large granules and droplets, apparently of a lipoidal nature, since they blacken with osmic acid and stain the same as the mitochondria in Schridde preparations. Further, in osmicated sections these granules are not removed by turpentine as fat droplets usually are. Figs. 15 and 16 show two cells in which the mitochondria (*M.*) are swelling up to form these bodies (*Ms.*). Noëlt† has described a similar transformation of mitochondria into plast like bodies in liver cells. The significance of this process in the thyroid cells, however, is not clear.

* Cramer and Ludford, 'J. Physiol.', vol. 61, p. 398 (1926).

† Noëlt, 'Arch. d'Anat. Microsc.', vol. 19, p. 1 (1923).



FIGS. 7-12.—Golgi apparatus in thyroid cells from a case of exophthalmic goitre in the mouse. *E.*, erythrocyte within blood capillary; *G.*, Golgi apparatus; *Gn.*, Golgi apparatus in normal position between the nucleus and the lumen of the vesicle; *Gr.*, Golgi apparatus reversed in polarity and directed towards a blood capillary; *S.*, secretion droplets.



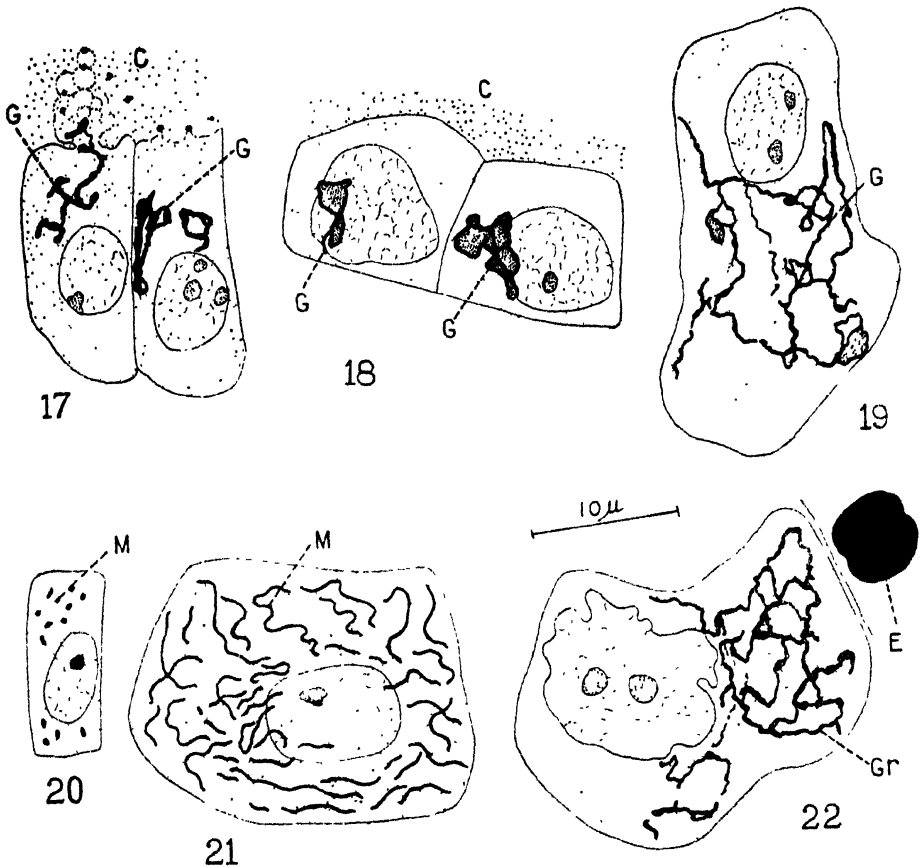
FIGS. 13-16.—Mitochondria in thyroid gland cells from a case of exophthalmic goitre in the mouse. *E.*, erythrocyte within blood capillary; *M.*, mitochondria; *Ms.*, swollen mitochondria.

Secretion in the Thyroid Cells in Exophthalmic Goitre in Man.

Our human material presents the typical histological features of exophthalmic goitre. The thyroid cells show the same degree of enlargement as in the case in the mouse. Some idea of the variation in the size of the cells can be obtained by a glance at the figs. 17 to 22. These are drawn from sections of the thyroids in two separate cases—figs. 17, 19, and 22 from one, figs. 18, 20, and 21 from the other.

The wide variation in the size of the mitochondria is seen in figs. 20 and 21. Fig. 20 represents a relatively inactive cell with granular mitochondria (*M.*), while fig. 21 is the stage of active secretion formation with long filamentous mitochondria (*M.*).

The Golgi apparatus shows a corresponding variation in size. Fig. 17 represents two cells with the apparatus (*G.*) in its normal position between the nucleus and the colloid. The cell on the left is discharging its secretion into the lumen of the vesicle. Fig. 18 shows two cells with the Golgi apparatus (*G.*) moved round, roughly through 90°. Figs. 19 and 22 show cells with the apparatus (*G.*) considerably enlarged and directed towards capillaries. The fact that we very rarely find secretion droplets forming in relationship with the



FIGS. 17-22.—Thyroid gland cells from cases of exophthalmic goitre in man. C., colloid; E., erythrocyte within blood capillary; G., Golgi apparatus; Gr., Golgi apparatus reversed in polarity; M., mitochondria.

Golgi apparatus in such cells, we attribute to the iodine administered to the patients before the operative removal of the glands.

Since our figures are drawn at a considerable magnification we have included a photomicrograph by Dr. G. S. Sansom, in support of our observations. Fig. 1, Plate 1, shows cells bordering a vesicle containing colloid (C.). One cell has the apparatus (Gn.) in the normal position, *i.e.*, directed towards the colloid. The cell immediately above has the apparatus (Gr.) completely reversed and directed towards a capillary (E.). The intermediate position of the apparatus (Gi.) is represented in the next cell above, while the cell at the bottom right-hand corner of the photograph has the apparatus (Ge.) in an equatorial position around the nucleus.

Summary and Conclusions.

From a cytological study of the thyroid gland in exophthalmic goitre in the mouse and in man it has been found—

- (i) There is considerable enlargement of the mitochondria (figs. 13, 14 and 20, 21) and of the Golgi apparatus (figs. 7, 8 and 17, 19)—a condition characteristic of intense secretory activity.
- (ii) The polarity of the Golgi apparatus is frequently reversed (figs. 7, 8 and 19, 22).
- (iii) The secretion droplets formed in association with the reversed apparatus, in the case of the mouse, are discharged directly into blood capillaries (figs. 9, 10 and 11).

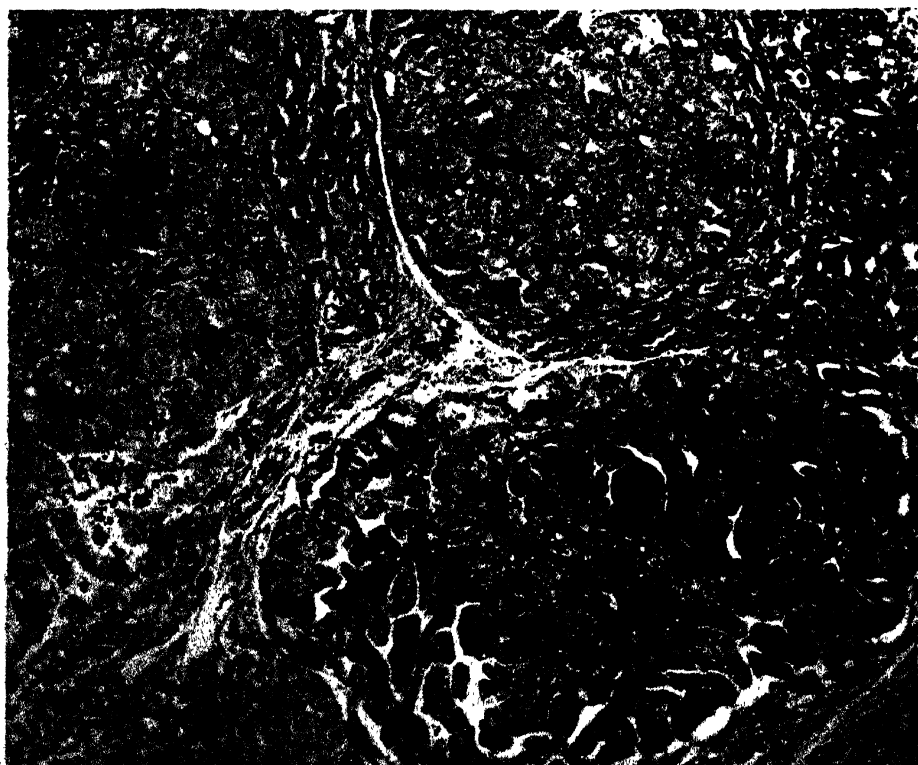
Secretion in the thyroid gland in exophthalmic goitre differs, therefore, from that in the normal active gland, in the direction in which the specific secretion is discharged. In the normal gland the secretion is discharged first into the lumen of the vesicle, in exophthalmic goitre there is a direct discharge into the blood capillaries.

It is difficult to believe that this difference in the mechanism of secretion of the normal and of the abnormal thyroid gland is without significance. Indeed the fact alone is significant that the cells of the normal gland pass out their specific secretion first into the alveolar lumen—that is to say, in a direction opposite to that of its ultimate destination, namely, the blood stream—and that they obstinately adhere to that roundabout method during prolonged activity. In this way the passage of the specific hormone from the cell to the blood stream is sharply divided into two separate phases which can be separately controlled. There is, moreover, evidence that the so-called “colloid” of the thyroid gland is not a homogeneous material, and that it does not pass uniformly from the alveolar lumen into the blood stream. It is quite conceivable, therefore, that some chemical change or interaction may take place in the alveolar lumen between the different materials which make up the so-called “colloid.”

On either view the direct passages of the specific hormone from the thyroid cell into the blood stream, which occurs in exophthalmic goitre, must produce an abnormal condition. The mechanism which controls the passage of the hormone into the circulation is no longer operative. If, moreover, the last-mentioned view is correct, the hormone which in exophthalmic goitre passes into the blood stream directly from the cell may be chemically different from that which in the normal gland passes from the alveolar lumen. The sug-



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gestion has been made repeatedly* that the condition of exophthalmic goitre is the result, not merely of an increased secretion of the thyroid hormone, but of the increased secretion of a slightly different hormone. The deviation from the normal mechanism of secretion, described in this communication, may therefore be regarded as the pathological alteration in the functional activity of the thyroid gland which underlies the condition of exophthalmic goitre.

DESCRIPTION OF PLATE I.

FIG. 1.—Variations in the position of the Golgi apparatus in thyroid cells from a case of exophthalmic goitre in man ($\times 1200$). *Cl.*, colloid; *E.*, blood capillary; *Ge.*, Golgi apparatus in an equatorial position around the nucleus; *Gi.*, Golgi apparatus moved round to the side of the nucleus; *Gn.*, Golgi apparatus in normal position; *Gr.*, Golgi apparatus reversed in polarity.

FIG. 2.—General low power view of the thyroid gland from a case of exophthalmic goitre in the mouse.

The Diffusion of Oxygen and Lactic Acid through Tissues.

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(From the Department of Physiology and Biochemistry, University College, London.)

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* See, for instance, Williamson and Pearse, 'Jour. of Path. and Bact.,' vol. 28, p. 361 (1925).

† The numbers of the equations and figures are shown in brackets, as also are (in Roman numerals) the numbers of the tables.

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INTRODUCTION.

The diffusion of dissolved substances through cells and tissues is a determining factor in many vital processes. The slowness of diffusion on the scale of ordinary sensible objects gives to the unaided imagination an imperfect realisation of its speed and importance in systems of the dimensions of the living cell. The diffusion constant k is expressed in terms of the number of unit quantities of substance which diffuse per minute across an area of 1 sq. cm. in a gradient of concentration per cm. of 1 unit quantity per c.c. For aqueous solutions of ordinary substances k is usually of the order of 2 to 10 times 10^{-4} . The diffusion constant is of the dimensions L^2T^{-1} , 2 in length, -1 in time. Expressing it in units of 1 μ (0.0001 cm.) instead of 1 cm., and of 1 σ (0.001 sec.) instead of minutes, k is of the order of unity, instead of a multiple of 10^{-4} . Thus the diffusion constant is a fairly large quantity for systems involving distances of the order of 1 μ and times of the order of 1 σ .

A cylinder 1 cm. in diameter composed of material similar to frog's nerve, if suddenly placed in oxygen, would take 185 minutes to attain 90 per cent. of its full saturation with that gas. An actual nerve 0.7 mm. thick would take 54 seconds for the same stage of saturation to be reached. A single nerve fibre 7 μ thick would take only 5.4 σ . Again, the rapidity of diffusion attainable in systems of small dimensions is the basis of the capillary circulation, and therewith of the whole design of the larger animals; and the rate at which diffusion can supply oxygen to a fatigued muscle for the removal of lactic acid is an important factor in determining the speed at which recovery can occur.

In another field, that of experimental methods available in physiology, the factor of diffusion is often important. The rate at which oxygen can pass into an experimental object, *e.g.*, a muscle, or a strip of tissue as employed by Warburg, and the depth to which it can penetrate; the speed with which phosphate, creatine, salts, carbon dioxide, or lactic acid can pass out; the velocity with which the recovered zone advances in a fatigued muscle suspended in oxygen or air; the lowest oxygen pressure which will maintain a tissue of given size in its normal condition; the time taken for the oxygen dissolved in a nerve to diffuse out when the nerve is suddenly immersed in pure nitrogen; such factors, of which unaided common-sense gives but the faintest indication, are matters often of great moment in the quantitative investigation of the chemical dynamics of living tissues.

Not many problems in diffusion can be completely solved in mathematical

terms. We are limited to certain special cases, for example the infinite plane sheet of finite thickness, the semi infinite solid, and the cylinder ; and even here a solution is generally available only for certain particular conditions. Many, however, of the objects with which physiologists have to deal conform approximately to one or other of these special cases ; and their solution will provide, if not a complete answer, at any rate an indication of the importance and range of the diffusion factor. In particular, the phenomena of the "steady state" dealt with in Part I have an obvious bearing on certain matters of general physiological interest.

In dealing with the kinetics of the diffusion process, considerations of dimensions alone will often provide valuable information. The diffusion constant k being of dimensions L^2T^{-1} , we may expect to find equations of the type $\frac{x}{\sqrt{kt}} = \text{a constant}$ for certain specified conditions, where x is a distance (e.g., the depth of the recovered zone in a fatigued muscle placed in oxygen) and t is time. The quantity Q of a substance which has diffused in or out in time t per sq. cm. of a surface (ML^{-2}) must bear to c , the concentration (ML^{-3}) which determines it, a ratio which must be of dimensions (L), and therefore proportional to \sqrt{kt} ; we must therefore be able to write, for any specified conditions,

$$Q \propto c\sqrt{kt}.$$

In the case of diffusion from a semi-infinite solid the exact formula can be shown to be

$$Q = 2c\sqrt{kt/\pi},$$

and the same ratio $2/\sqrt{\pi}$ must hold approximately for other cases, provided that t be not too great. Such general relations will continually be found ; and to realise their occurrence will often be a help in looking for, or recalling, a solution.

PART I.—DIFFUSION DURING A "STEADY STATE."

Various important problems and methods in physiology involve the condition of a "steady state" depending upon a uniform diffusion of some dissolved substance into or out from a tissue. For example, a muscle suspended in oxygen-free Ringer's solution produces lactic acid slowly at a constant rate, and this diffuses outwards into the Ringer's solution ; given the shape and dimensions of the muscle, calculate the final concentration of lactic acid attained inside. Again, a nerve is placed in nitrogen containing a known small pro-

portion of oxygen ; given the oxygen requirement of the tissue per minute, calculate what fraction of the nerve's cross-section receives an adequate supply. In special cases solutions of such problems have been given already, *e.g.*, by Krogh (1), Warburg (2), Gerard (3) and Fenn (4). The matter will be considered here more generally.

§ 1. SOLID BOUNDED BY AN INFINITE PLANE.

(A) *Diffusion of Oxygen from a Gaseous or Liquid Phase in which its Concentration is maintained Constant, into a Solid in which it is used up by Metabolic Processes at a Constant Rate.*

Let y be the concentration of oxygen (c.c. at N.T.P. per c.c. of tissue) at any point inside the tissue distant x from the surface. Let k be the diffusion constant (cm.²/min.), and y_0 the concentration of oxygen constantly maintained at $x = 0$. Let a be the rate (c.c. at N.T.P. per c.c. of tissue per minute) at which oxygen is used up by the tissue. The equation determining the diffusion can then be derived as follows.

In fig. 1 the rate of diffusion to the right across any given square centimetre parallel to the surface is $-k \frac{dy}{dx}$; if $-\frac{dy}{dx}$ were constant (*i.e.*, if the gradient of concentration were uniform) there would be no accumulation at any point ; if, however, $-\frac{dy}{dx}$ diminishes as x increases more oxygen reaches any given region than passes on from it, and oxygen tends to accumulate in that region. The measure of the rate of accumulation is $k \frac{d^2y}{dx^2}$. This tendency to accumulate is met, either by an increase in the concentration, or by a utilisation of the oxygen by the tissue. Expressed mathematically, the diffusion equation is

$$\frac{dy}{dt} + a = k \frac{d^2y}{dx^2}. \quad (1)$$

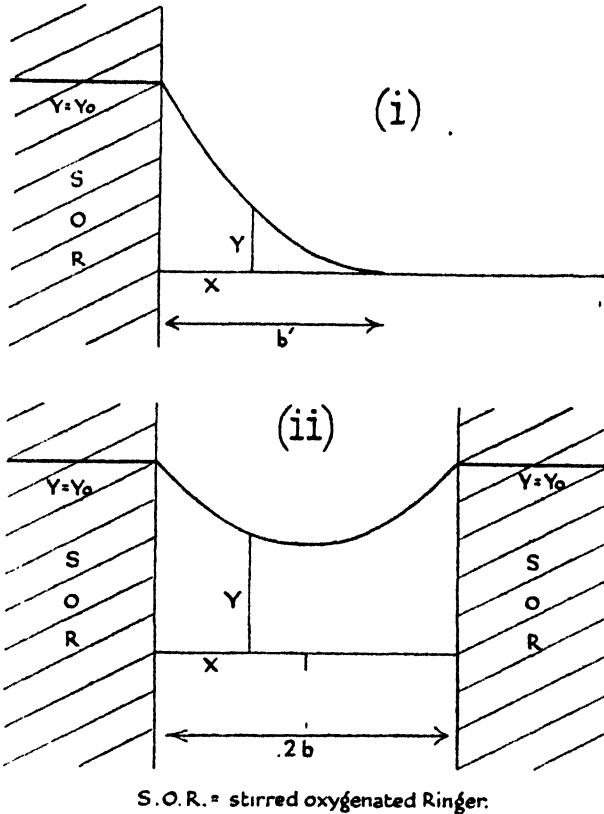
Since we are dealing here with a steady state, $\frac{dy}{dt} = 0$, and we have

$$a = k \frac{d^2y}{dx^2}. \quad (2)$$

The solution of this equation is,

$$y = ax^2/2k + Bx + y_0$$

where B is a constant to be determined ; at $x = 0$, $y = y_0$ as it should.



S.O.R. = stirred oxygenated Ringer.

FIG. 1.—Diffusion of oxygen into a plane sheet of tissue during a "steady state": y = oxygen concentration; x = distance from surface; y_0 = constant concentration maintained at surface. (i) Tissue of infinite thickness, or of thickness greater than that (b') which oxygen can completely penetrate. (ii) Tissue of finite thickness ($2b$) completely penetrated by oxygen. It is assumed that the tissue consumes oxygen at a constant rate, and that lactic acid produced in the oxygen-free region of (i) cannot diffuse.

Now, if a be positive, y must diminish as we pass inwards, and a point must be reached at which $y = 0$. The concentration, however, can never be negative, so that at this point the diffusion flow must stop and therefore the concentration gradient become zero (see fig. 1(i)). Thus if $x = b'$ be the distance from the surface at which the concentration vanishes we must have,

$$y = 0 = ab'^2/2k + Bb' + y_0,$$

and

$$\frac{dy}{dx} = 0 = ab'/k + B.$$

Hence

$$B = -ab'/k \quad \text{and} \quad ab'^2/2k = y_0.$$

Thus

$$b' = \sqrt{2ky_0/a} \quad \text{and} \quad B = -\sqrt{2ay_0/k}.$$

Hence the solution we require is

$$y = ax^2/2k - x\sqrt{2ay_0/k} + y_0 \quad (3)$$

and the greatest thickness to which the oxygen penetrates is given by

$$b' = \sqrt{2ky_0/a}. \quad (4)$$

The total amount Q' of oxygen dissolved in the tissue, per sq. cm. of its surface, during the steady state, is $\int_0^{b'} y dx$, which on integrating (3) becomes

$$Q' = \frac{1}{3}y_0\sqrt{2ky_0/a} = y_0b'/3. \quad (5)$$

This is one-third of the full amount of oxygen which could be dissolved, in the absence of oxygen consumption, in a thickness b' of tissue.

If the tissue involved be limited by contact with an impermeable wall at $x = b$, or if it be of thickness $2b$ and exposed to oxygen on both sides, then two cases arise, according as b is greater or less than b' . If $b > b'$ the above solution (fig. 1 (i)) holds. If $b < b'$ then y does not attain zero at any point of the tissue, and the condition determining B is that there is no diffusion across the plane $x = b$, and therefore $dy/dx = 0$ at $x = b$ (fig. 1 (ii)). This gives

$$0 = \frac{dy}{dx} = ab/k + B, \quad B = -ab/k.$$

The solution is now

$$y = ax^2/2k - abx/k + y_0. \quad (6)$$

The total oxygen Q dissolved in the tissue from $x = 0$ to $x = b$, per sq. cm. of surface, during the steady state, is then

$$Q = y_0b - ab^2/3k. \quad (7)$$

The full amount which the muscle would dissolve if there were no consumption of oxygen is y_0b . Hence the fraction f of the full saturation amount actually dissolved is

$$f = 1 - ab^2/3ky_0, \quad (8)$$

which may be written

$$f = 1 - \frac{1}{3}b^2/b'^2 \quad (9)$$

where b' represents the greatest thickness which can be supplied with oxygen, as defined above.

(a) *Absolute Values for Frog's Muscle: the Diffusion Constant.*—According

to Krogh (5) the diffusion constant of oxygen through muscle at 20° C. is about 1.4×10^{-5} , and increases about 1 per cent. per 1° C. rise of temperature. Krogh's constant is expressed in c.c. of oxygen diffusing across 1 sq. cm. per minute, in a concentration gradient corresponding to a partial pressure gradient of 1 atmosphere per cm. The use of a constant expressed in this way is very convenient provided that we are concerned only with a steady state, or in certain other cases. In dealing, however, with the kinetics of diffusion, as we shall see below, it is advisable to adopt the usual system. If we employ Krogh's constant, y and y_0 are not concentrations in the ordinary sense, but partial pressures. In that case, in order to obtain actual concentrations or quantities we must multiply y , y' , Q or Q' by the solubility coefficient, which for oxygen at 20° C. may be taken as 0.031.

(b) *Critical Depth of a Resting Muscle Fully Supplied with Oxygen.*—The rate of oxygen consumption in a resting frog's muscle at 20° C. may be taken (6, p. 140) as about 0.0007 c.c. per c.c. of muscle per minute. If we consider a sheet of resting muscle exposed at 20° C. on one surface only to oxygen at a partial pressure of 1 atmosphere, we find from equation (4) the depth b' to which the oxygen penetrates,

$$b' = \sqrt{2 \times 1.4 \times 10^{-5} \times 1 / (7 \times 10^{-4})} = 0.2 \text{ cm.} = 2 \text{ mm.} \quad (\text{Oxygen}).$$

If the gas be air containing (say) 21 per cent. of oxygen we find similarly,

$$b' = 0.9 \text{ mm.} \quad (\text{Air}).$$

If the gas be ordinary cylinder nitrogen containing (say) 0.5 per cent. oxygen,

$$b' = 0.14 \text{ mm.} \quad (0.5 \text{ per cent. } O_2).$$

If the nitrogen be specially pure, containing (say) 0.01 per cent. oxygen,

$$b' = 0.02 \text{ mm.} \quad (0.01 \text{ per cent. } O_2).$$

Thus only about 1 muscle fibre on the outside surface will be supplied with oxygen at this low pressure.

At a higher temperature the value of k will be slightly greater but that of a much greater. At 37° C. if we take k as 1.7×10^{-5} and a as 34×10^{-4} , the values of b' will, in each case, be exactly one-half of those given above.

(c) *The Oxygen contained, per sq. cm. of Surface, in a Resting Sheet of Muscle.*
—(i) If the muscle be too thick to have its whole volume penetrated by oxygen we use equation (5) above,

$$Q' = y_0 b' / 3,$$

where b' has the values just given (but expressed in cm.). y_0 for 1 atmosphere partial pressure is 0.031. We find :—

Partial pressure of oxygen, atmospheres	1	0.21	0.005	0.0001
Cubic centimetres of oxygen dissolved per square centimetre of surface . .	2.06×10^{-3}	1.95×10^{-4}	7.2×10^{-7}	2.1×10^{-9}

(ii) When the muscle is thin enough to have its whole volume penetrated by oxygen we may use equation (9),

$$f = 1 - \frac{2}{3} b^2 / b'^2.$$

For oxygen at a partial pressure of 1 atmosphere b' , from above, is 2 mm. Hence a frog's sartorius muscle 0.7 mm. thick exposed at 20° C. to pure oxygen on one side only will contain, when it reaches a steady state, 92 per cent. of the full amount of oxygen it could dissolve at a partial pressure of 1 atmosphere throughout, *i.e.*, about 0.0285 c.c. per c.c. Exposed on both sides it would contain 98 per cent., *i.e.*, about 0.0304 c.c. per c.c. The same muscle exposed to air on one side only, for which $b' = 0.9$ mm., would contain about 40 per cent. of the amount it would dissolve at the partial pressure of oxygen in air; this is about 0.0026 c.c. per c.c. Exposed on both sides it would dissolve 85 per cent. of the full amount, 0.0055 c.c. per c.c. A much thicker muscle, *e.g.*, the semi-membranosus of the frog 2 mm. thick, exposed on one side only, would just be penetrated by oxygen at a partial pressure of 1 atmosphere; in air the oxygen would penetrate only 0.9 mm., less than half-way. In oxygen, the total amount of dissolved oxygen would be one-third of the full saturation amount, about 0.0103 c.c. per c.c.; in air it would be very small.

Such calculations throw considerable light upon the conditions necessary for a study of the recovery heat-production in muscle. A muscle on a thermopile is exposed on one side only. If stimulated it requires oxygen for recovery. If there be sufficient oxygen already dissolved in it, the recovery process can proceed at its own intrinsic rate, unaffected by the speed at which the necessary oxygen can be supplied by diffusion. A short tetanus (say $\frac{1}{4}$ second at 20° C.) gives an "initial" rise of temperature of about 0.02° C., a total heat (including recovery) of about 0.042 calorie per c.c. of tissue. This would require about 0.0084 c.c. of oxygen per c.c. Thus a sartorius muscle 0.7 mm. thick in a steady resting state in oxygen contains more than enough oxygen already dissolved in it to allow recovery to reach completion, without any

further supply from outside ; the employment of a still thinner muscle would give no serious advantage, since a muscle of this size already contains 92 per cent. of its full amount. On the other hand, in air, the amount of oxygen dissolved is only 0.0026 c.c., which is less than one-third of the quantity required for recovery even from this short stimulus. Clearly the time-course of recovery will now be chiefly determined by the speed of inward diffusion of oxygen. In the case of the thicker semi-membranosus muscle in oxygen the amount initially dissolved (0.0103 c.c. per c.c.) is slightly greater than the total amount required for recovery (0.0084), but it should be noted that the major portion of this is in the outer layers, and that there will be an inadequate supply in the inner layers. Even in pure oxygen therefore the observed time-course of the recovery process in a muscle 2 mm. thick will be affected by the slowness of diffusion. In air, recovery will be possible only in the outer layers.

The matter can be approached in another way. The amount of oxygen required for complete recovery from a contraction of the extent considered is 0.0084 c.c. per c.c. of muscle ; this, at 20° C., represents a partial pressure of 0.27 atmosphere. If recovery is to be completely unaffected by the speed of oxygen diffusion, the partial pressure must initially be greater everywhere than this quantity. The lowest pressure is on the innermost side, where the muscle rests against the thermopile, viz., at $x = b$. Substituting $x = b$ in equation (6) we find for the least oxygen partial pressure ($y_0 - ab^2/2k$). Substituting their values for a and k this is ($y_0 - 25b^2$). If $y_0 = 1$ and ($y_0 - 25b^2$) = 0.27 we have $b = 0.17$, so that 1.7 mm. is the greatest thickness allowable.

A single twitch, in contraction and recovery, gives about 0.007 calorie per gram, uses therefore about 0.0014 c.c. of oxygen per c.c. This represents a partial pressure of 4.5 per cent. of an atmosphere, so that the initial oxygen partial pressure on the inner edge must not be below this, if recovery is to be unaffected by the rate of oxygen supply. Putting ($y_0 - 25b^2$) = 0.045 we find the following values :—

$$y_0 = 1 \text{ (oxygen), } b = 1.95 \text{ mm.}$$

$$y_0 = 0.21 \text{ (air), } b = 0.81 \text{ mm.}$$

Thus 2 mm. is too thick, even for the case of a single twitch in oxygen ; 0.81 mm. is the thickest muscle for a single twitch in air.

We see from such calculations that if we are, by myothermic methods, to study the time-course and extent of the recovery process after activity, and

not allow these to be obscured by an insufficient supply of oxygen, we must work :—

- (a) with thin muscles,
- (b) in oxygen rather than air, and
- (c) with not too prolonged contractions.

We must allow, moreover, an approximate return to a steady state to be attained after each observation. These are the very conditions which have been found, experimentally, to yield the most consistent results; indeed, it is obvious from the above calculations, that a thicker muscle, such as a gastrocnemius or a semi-membranosus, or any ordinary muscle in air, rather than oxygen, will have its recovery process so protracted by the slow diffusion of the requisite oxygen as to be scarcely susceptible of accurate study. The only way to follow the recovery process is to employ a muscle so thin, and exposed to such a high concentration of oxygen, that sufficient oxygen will be already dissolved in the water of the muscle to allow the recovery process to reach completion, without waiting for an extra supply by diffusion. If this precaution be neglected, the results are bound to be imperfect.

(d) *The Oxygen Supply to an Active Sheet of Muscle.*—In an active muscle the oxygen requirement is much greater than at rest. The greatest thickness b' for an adequate supply of oxygen is related to the oxygen requirement a by equation (4). $b' = \sqrt{2ky_0/a}$. Consider a frog's muscle 0.5 mm. thick (e.g., the small sartorius of an English frog) executing single twitches at regular intervals at 20° C., when exposed to oxygen on one side only. We require to find the highest degree of activity allowable. From equation (4) $a = 2ky_0/b'^2 = 1.12 \times 10^{-2}$ c.c. of oxygen per c.c. per minute. Subtracting the resting oxygen consumption this leaves 1.05×10^{-2} c.c. per minute available for activity. Assuming 1 c.c. of oxygen to be the equivalent of 5 calories this means that the greatest rate at which heat can be liberated continuously by such a muscle is about 5.2×10^{-2} calorie per c.c. per minute. Now the single twitch of a muscle liberates (in initial and recovery processes together) about 7×10^{-3} calorie per c.c. Thus our muscle should be able to carry out regularly one twitch every 8 seconds, without acquiring an oxygen debt. If it were twice as thick only one twitch every 40 seconds would be allowable; if it were half as thick, one twitch every 2 seconds. If the gas were air instead of oxygen the number of twitches possible would be only about 1 in 50 seconds. If the muscle were exposed to oxygen on both sides, the stimuli might be rather

more* than four times as frequent. Thus in the case of a muscle 0.25 mm. thick exposed to pure oxygen on both sides, more than 2 twitches per second would be possible, without incurring an increasing oxygen debt; while in a muscle more than 4 mm. thick similarly exposed, the resting metabolism alone would gradually lead to fatigue.

(B) *Diffusion of Lactic Acid into a Liquid Phase in which its Concentration remains Constant, from a Solid in which it is formed by Metabolic Processes at a Constant Rate.*

This case is the exact converse of that dealt with in (A) above, with the sign of α changed. For α write $-\alpha$: then equation (2) becomes,

$$-\alpha = k' \frac{d^2 y'}{dx^2} \quad (10)$$

where y' is the concentration of lactic acid and k' its diffusion constant. The concentration y' must now increase as we pass inwards, and no steady state will occur unless the system be limited in the direction of x . Let the system be in contact with an impermeable wall at $x = b$, or let it be of thickness $2b$ and exposed on both sides; then the solution, as in (6) above, is,

$$y' = -\alpha x^2/2k' + \alpha bx/k' + y'_0 \quad (11)$$

and the total amount dissolved, during the steady state, is, as in (7) above,

$$Q = y'_0 b + \alpha b^3/3k'. \quad (12)$$

Consider the case of the lactic acid concentration in a resting muscle suspended in a large bulk of stirred oxygen-free Ringer's solution for which $y'_0 = 0$. The diffusion "constant" of lactic acid through muscle has recently been studied by Eggleton, Eggleton and Hill (7). It is not independent of the lactic acid concentration, decreasing in fatigued muscles to a very low value of about 5.5×10^{-6} ; it depends presumably upon the amount of lymph interspaces in the tissue. With a relatively unfatigued muscle it is of the order of 6×10^{-5} . For the sake of illustrating the argument we will often assume the latter value; the coefficient of diffusion of lactic acid through muscle is not, however, sufficiently constant to allow us to make any very exact deductions by its use. In some cases, however (see e.g., (C), p. 55 below), results involving the diffusion of lactic acid can be deduced without a knowledge of its diffusion constant. The value of α may be calculated from the rate of resting oxygen consumption at 20° C., assuming that the formation

* *More*, because taking account of the resting metabolism as we have done slightly distorts the simple square root relation.

of 4.8 grams. of lactic acid is prevented by the oxidation of 1 grm. of lactic acid (see (8)) at the expense of 22.4/30 litres of oxygen. This makes 1 c.c. O_2 used $\equiv 6.43$ mgrms. lactic acid formed, so that 7×10^{-4} c.c. O_2 used per minute $\equiv 4.5 \times 10^{-6}$ grm. lactic acid formed per minute, and for frog's muscle at $20^\circ C.$ $\alpha = 4.5 \times 10^{-6}$. Hence

$$\alpha/k' = 4.5 \times 10^{-6}/6 \times 10^{-5} = 7.5 \times 10^{-2}.$$

Equation (11) becomes therefore

$$y' = 0.075 bx - 0.037x^2.$$

It is of interest to determine the maximum concentration attained, which will be where $x = b$. At $x = b$

$$y' = 0.037b^2,$$

and if $b = 0.075$, for a muscle 0.75 mm. thick exposed on one side, or 1.5 mm. thick exposed on both sides,

$$y'_{x=b} = 2.1 \times 10^{-4}.$$

Thus the greatest concentration attained will be about 0.02 per cent. In a muscle 2 mm. thick exposed on one side ($b = 0.2$) the greatest concentration similarly calculated is 0.15 per cent. *Thus considerable concentrations of lactic acid may arise in the interior of thick muscles even when suspended in Ringer's solution.*

The total lactic acid inside the muscle, per sq. cm. of surface is, from (12), $xb^3/3k'$, or averaged per c.c. $\alpha b^2/3k'$. Substituting the above value for α/k' we find the total amount to be $0.025b^3$; for a muscle 0.075 cm. thick this means an average lactic acid concentration of 0.014 per cent., for a muscle 0.2 cm. thick one of 0.1 per cent., for one 0.3 cm. thick one of 0.22 per cent. We see therefore that contact with Ringer's solution on the surface cannot be expected to prevent the accumulation of lactic acid inside muscles more than about 1 mm. thick. A frog's sartorius is thin enough to have its lactic acid removed by diffusion into Ringer's solution in which it is suspended; a gastrocnemius muscle is not.

It is interesting to inquire how frequently a thin plane sheet of muscle could be caused to twitch, while in oxygen-free Ringer's solution, without the accumulation of considerable quantities of lactic acid inside it. Let $2b$ be the thickness, the muscle being exposed to Ringer's solution on both sides. A thin sartorius muscle of the frog is about 0.5 mm. thick; thus $b = 0.025$. A single twitch gives a rise of temperature of about $0.0035^\circ C.$, which, with 385 calories per grm. of lactic acid liberated, means about 10^{-5} grm. of lactic

acid liberated per c.c. of muscle. If the muscle give n twitches per minute it liberates $n \times 10^{-5}$ grm. of lactic acid per c.c. per minute, so that $\alpha = n \times 10^{-5}$. $\alpha/3k'$ therefore has the value $n/18$, so that if $b = 2.5 \times 10^{-2}$ the value of $\alpha b^2/3k'$ is $n \times 3.5 \times 10^{-5}$ which is $n \times 3.5 \times 10^{-3}$ per cent. Thus 60 twitches per minute should lead ultimately to an average lactic acid concentration of 0.21 per cent., which would cause considerable fatigue; 6 twitches per minute should cause an average concentration of only 0.021 per cent., a very low value, insufficient to cause fatigue. The greatest concentration, in the middle of the muscle at $x = b$, is from (11) $\alpha b^2/2k'$: with 60 twitches per minute this is, for the same case, 0.31 per cent., about the fatigue maximum of frog's muscle; thus this rate of stimulation should lead definitely to fatigue. On the other hand the greatest concentration at $x = b$ for 6 twitches per minute is only 0.031 per cent., a very low value. Thus, if lactic acid accumulation were the only cause of fatigue a thin sartorius muscle suspended in Ringer's solution would be able to give 6 twitches per minute until the whole of its supply of lactic-acid-producing substance was exhausted.

In the case of thicker muscles (neglecting the resting production of lactic acid), since the maximum concentration attained at a given frequency of twitch varies as the square of the thickness, the greatest possible frequency falls rapidly as the thickness increases. In a muscle 1 mm. thick exposed to Ringer's solution on both sides, the maximum concentration attained at 6 twitches per minute is 0.124 per cent., enough to produce perceptible fatigue; in a muscle 2 mm. thick the maximum concentration even at 3 twitches per minute is 0.25 per cent., enough to cause almost complete fatigue.

Thus the effect of Ringer's solution in maintaining the condition of a muscle excited anaerobically should apply only to very thin muscles, and even then only to relatively low rates of stimulation. This is even more the case owing to the fact that at higher concentrations of lactic acid, greater (say) than 0.15 per cent., the diffusion constant, according to Eggleton, Eggleton and Hill (7), falls considerably, so tending to cause an increased accumulation in the excited tissue. To get the full effect of diffusion into the surrounding Ringer the muscle should be exceedingly thin, and the frequency of stimulation low. An interesting field of work on the onset of fatigue under anaerobic conditions is suggested by these calculations.

(C) *The Simultaneous Diffusion of Oxygen and Lactic Acid in the Steady State.*

If two chemical substances neutralise one another, as for example an acid and alkali, a type of steady state may occur in which there is a surface of neutrality: on the one side there is an excess of one substance, on the other side of the other. An important case arises in physiology when a tissue is so thick that the supply of oxygen by diffusion is insufficient to penetrate the whole mass; lactic acid is then formed in the part to which the oxygen cannot penetrate, and diffuses towards the oxygen region, where it is removed, a steady state finally being attained in which lactic acid occurs on one side (fig. 2) of the neutral surface and oxygen on the other.

Consider a plane sheet of tissue of thickness b subjected to oxygen at partial

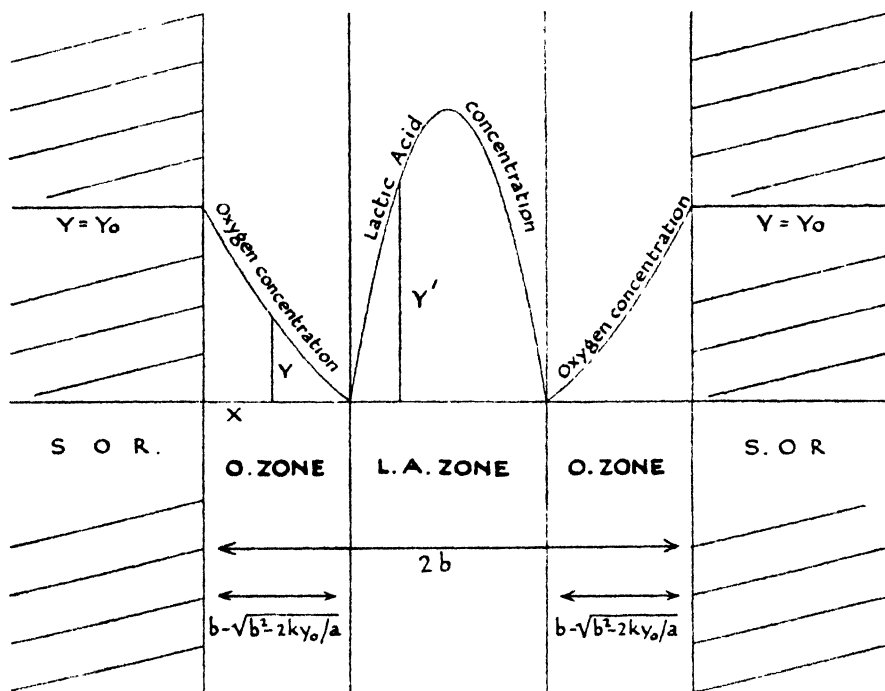


FIG. 2.—Diffusion of oxygen and lactic acid in a plane sheet of tissue during a steady state: y = oxygen concentration at any point in the outer zone distant x from surface; y_0 = constant oxygen concentration maintained at surface; y' = lactic acid concentration in the inner zone; $2b$ = thickness of tissue; k = diffusion constant of oxygen. It is assumed that the tissue consumes oxygen at a constant rate a in the outer zone, and produces lactic acid at an equivalent rate in the inner zone. Note that the depth of the oxygen zone is independent of the diffusion constant of lactic acid. S.O.R. = Stirred Oxygenated Ringer.

pressure y_0 on the left, and placed against an impenetrable wall on the right ; or a sheet of thickness $2b$ exposed to oxygen on both sides. Let the tissue be thicker than $b' = \sqrt{2ky_0/a}$ (equation (4) above) so that oxygen is unable to penetrate the whole, and lactic acid is produced on the right. Let y be the concentration (partial pressure) of oxygen on the left ; y' that of lactic acid on the right. Let a be the rate of oxygen utilisation per c.c. of tissue on the left ; α that of lactic acid formation on the right. Let k and k' be the respective values of the diffusion constants. Then the diffusion equations are, during the steady state :—

$$a = k \frac{d^2 y}{dx^2} \quad (\text{oxygen on the left}) \quad (2)$$

$$-\alpha = k' \frac{d^2 y'}{dx^2} \quad (\text{lactic acid on the right}) \quad (10)$$

The boundary conditions are :—

$$(a) \ y = y_0 \text{ at } x = 0, \quad (b) \ \frac{dy'}{dx} = 0 \text{ at } x = b.$$

(c) y and y' must become zero at the same value of x ; it is realised that a region of finite thickness will actually be required to complete the oxidative removal of the lactic acid diffusing from the right, but for the sake of the mathematical treatment it is supposed that this region is very thin ; no great error should result from this simplification.

(d) At the plane where y and y' simultaneously vanish the oxygen diffusing to the right is sufficient to complete the oxidative removal of the lactic acid diffusing to the left ; expressed mathematically

$$\begin{aligned} -k \frac{dy}{dx} &= \text{number of c.c. of oxygen required to effect the oxidative removal} \\ &\quad \text{of 1 grm. of lactic acid,} \\ &= \alpha/\alpha. \end{aligned}$$

It can easily be shown by trial that the following expressions satisfy the differential equations on the one hand, and the boundary conditions (a) to (d) on the other :

$$y = ax^2/2k - abx/k + y_0 \quad (13)$$

$$y' = -\alpha x^2/2k' + \alpha bx/k' - y_0 k\alpha/k'a. \quad (14)$$

It will be noted that equation (13), though in form the same as (6) above, refers to a case where $b > b'$; when no account was taken of the diffusion of lactic acid this case was governed by equation (3), not by equation (6).

Equation (13) applies, for the case of oxygen, to the region on the left; equation (14), for the case of lactic acid, to the region on the right. The plane at which the oxygen and lactic acid meet, *i.e.*, where $y = 0$ and $y' = 0$, is given by the equation

$$x = b - \sqrt{b^2 - 2ky_0/a}.$$

Its position is independent of the diffusion constant of the lactic acid. If the latter be less the gradient of concentration in the steady state will be greater and so compensate for it. The oxygen penetrates the whole muscle if $b \geq \sqrt{2ky_0/a}$, which is the condition we obtained in (4) above. Calling this critical depth b' we find, for the depth at which oxygen and lactic acid meet,

$$x_0 = b - \sqrt{b^2 - b'^2}. \quad (15)$$

We found above for a plane sheet of resting muscle, exposed on one side only to oxygen at one atmosphere pressure, a value of $b' = 2$ mm. For $b = 2.5$ mm., $x_0 = 1.0$ mm.; for $b = 3$ mm., $x_0 = 0.76$ mm.; for $b = 4$ mm., $x_0 = 0.54$ mm. Thus the depth to which oxygen penetrates rapidly diminishes as the thickness of the muscle increases. In a thick muscle only quite a thin layer on the surface will contain any oxygen.

The expression for the lactic acid concentration given in equation (14) above is identical with that of the earlier equation (11) apart from the constant. Equation (7) in the form

$$y' = -\alpha r^2/2k' + \alpha br/k',$$

applies to the steady state of a muscle producing lactic acid when suspended in oxygen-free Ringer's solution. The effect of adding oxygen to the Ringer's solution at partial pressure y_0 is to make an outer zone free of lactic acid of depth given by equation (15), and to lower the concentration throughout the remainder of the tissue by the amount $y_0 k\alpha/k'a$, the constant of equation (14). It will be noted that the absolute values of a and α do not occur in this expression, only their ratio; the lowering, therefore, of lactic acid concentration produced by oxygen in the Ringer is the same, whatever be the temperature or the state of activity of the tissue (assuming k and k' to be similarly affected by temperature and activity). Taking the values of the constants employed for a resting muscle at 20°C ., *viz.*, $k = 1.4 \times 10^{-5}$, $k' = 6 \times 10^{-5}$, $a = 7 \times 10^{-4}$, $\alpha = 4.5 \times 10^{-6}$, we find $k\alpha/k'a = 1.5 \times 10^{-3}$. Thus the effect of saturating the Ringer with oxygen ($y_0 = 1$) is to diminish the lactic acid concentration throughout the tissue, *either* to zero in an outer zone, *or* by 0.15 per cent. in an inner zone. The effect of saturating with air ($y_0 = 0.21$) is

similarly to diminish the lactic acid concentration, *either* to zero, *or* by 0.03 per cent.

§ 2. SOLID BOUNDED BY AN INFINITELY LONG CYLINDER.

Some tissues, *e.g.*, nerves, can be treated as long circular cylinders. The solution, therefore, of the cylindrical problem, for the case of a steady state, is of interest.

(A) *Diffusion of Oxygen from a Gaseous or Liquid Phase in which its Concentration is maintained Constant, into a Cylindrical Solid in which it is used up by Metabolic Processes at a Constant Rate.*

Let r be the distance measured from the axis of the cylinder of a point at which the concentration is y ; r_0 the radius of the cylinder, y_0 the concentration constantly maintained at $r = r_0$, k the diffusion constant and a the rate per c.c. of tissue per minute, at which the oxygen is consumed.

The diffusion equation is

$$\frac{dy}{dt} + a = \frac{k}{r} \frac{d}{dr} \left(r \frac{dy}{dr} \right). \quad (21)$$

For a steady state $dy/dt = 0$, and we have

$$a = \frac{k}{r} \frac{d}{dr} \left(r \frac{dy}{dr} \right). \quad (22)$$

The solution of this equation is

$$y = ar^2/4k + B \log r + E, \quad (23)$$

where B and E are constants depending on the boundary conditions.

Assume first that the oxygen penetrates the whole cylinder. Then since the concentration at the axis is finite B must be zero, while since $y = y_0$ at $r = r_0$

$$E = y_0 - ar_0^2/4k.$$

In this case the solution is

$$y = y_0 - a(r_0^2 - r^2)/4k, \quad (24)$$

and the concentration at $r = 0$ is $y = y_0 - ar_0^2/4k$. The thickest cylinder which the oxygen will penetrate is that in which $y = 0$ at $r = 0$. Its radius is given by

$$r_0' = \sqrt{4ky_0/a}. \quad (25)$$

This is the same as equation (4) above, but with 4 substituted for 2 in the numerator. The total quantity Q of oxygen contained in 1 cm. length of the cylinder, in the case when oxygen penetrates the whole ($r_0' < \sqrt{4ky_0/a}$) is

$$\int_0^{r_0'} 2y\pi r dr,$$

which becomes

$$Q = \pi r_0'^2 [y_0 - ar_0'^2/8k]. \quad (26)$$

If there were no metabolism this would be

$$Q = \pi r_0^2 y_0.$$

Hence the fraction f of the full saturation amount actually dissolved is

$$f = 1 - ar_0'^2/8ky_0 \quad (27)$$

which may be written

$$f = 1 - r_0'^2/2r_0'^2 \quad (28)$$

where r' represents the greatest radius of a cylinder which can be fully supplied with oxygen.

If the oxygen does not reach the axis of the cylinder, but attains some constant concentration y_1 at $r = r_1$, the constant B in equation (23) is not zero. The boundary conditions are then $y = y_0$ at $r = r_0$, $y = y_1$ at $r = r_1$. Hence

$$y_0 = ar_0^2/4k + B \log r_0 + E$$

$$y_1 = ar_1^2/4k + B \log r_1 + E.$$

This gives

$$B = \frac{y_0 - y_1 - a(r_0^2 - r_1^2)/4k}{\log r_0/r_1}$$

and

$$E = \frac{y_1 \log r_0 - y_0 \log r_1 - a(r_1^2 \log r_0 - r_0^2 \log r_1)/4k}{\log r_0/r_1}.$$

Equation (23) is then the solution with these values of B and E .

It should be noticed that r_1 may be greater or less than r_0 . An equation for an analogous case has been given by Krogh (1). A tissue is intersected by blood-capillaries of radius r_0 , and each capillary supplies oxygen to a region of radius r_1 around it. Outward diffusion therefore stops at $r = r_1$, so that $(dy/dr)_{r=r_1} = 0$, while $y = y_0$ at $r = r_0$.

We have

$$y = ar^2/4k + B \log r + E$$

and

$$y_0 = ar_0^2/4k + B \log r_0 + E.$$

Hence

$$y = y_0 + a(r^2 - r_0^2)/4k + B \log r/r_0.$$

Also $dy/dr = 0$ at $r = r_1$: hence

$$0 = ar_1/2k + B/r_1, \quad B = -ar_1^2/2k.$$

Hence

$$y = y_0 - a[r_1^2 \log r^2/r_0^2 - r^2 + r_0^2]/4k \quad (29)$$

The value of y at $r = r_1$ is then

$$y_1 = y_0 - a[r_1^2 \log r_1^2/r_0^2 - r_1^2 + r_0^2]/4k. \quad (30)$$

These equations apply equally to the case of diffusion inwards from $r = r_0$ into a cylinder which is bounded by an impermeable membrane at $r = r_1$. Here, of course, r and r_1 are $< r_0$. For a further discussion of this case see below, Part III, § 2, Case III.

An interesting case is that of a nerve only partially supplied with oxygen by diffusion. Let us assume that at $r = r_0$, $y = y_0$, while at $r = r_1$, $y = 0$, and diffusion stops at this spot. We have then $dy/dr = 0$ at $r = r_1$. We must imagine that in spite of an oxygen debt in the interior no diffusion outwards of any substance similar to lactic acid occurs; diffusion ends when y becomes zero. We then have, employing equation (23),

$$\left. \frac{dy}{dr} \right|_{r=r_1} = 0 = ar_1/2k + B/r_1.$$

Therefore

$$B = -ar_1^2/2k.$$

Hence

$$y_0 = ar_0^2/4k - (ar_1^2 \log r_0)/2k + E$$

$$0 = ar_1^2/4k - (ar_1^2 \log r_1)/2k + E.$$

Subtracting,

$$\begin{aligned} y_0 &= a[r_0^2 - r_1^2 - r_1^2 \log r_0^2/r_1^2]/4k \\ &= ar_0^2[1 - r_1^2/r_0^2 - (r_1^2/r_0^2) \log r_0^2/r_1^2]/4k. \end{aligned}$$

Put $r_1^2/r_0^2 = 1 - \theta$, so that θ is the fraction of the whole cross-section of the nerve which is supplied with oxygen. We then have

$$y_0 = (ar_0^2/4k) \left[\theta - (1 - \theta) \log_e \left(\frac{1}{1 - \theta} \right) \right]. \quad (31)$$

This equation can be solved by constructing a curve for

$$\left[\theta - (1 - \theta) \log_e \left(\frac{1}{1 - \theta} \right) \right]$$

as a function of θ . If θ be small we may write $\log\left(\frac{1}{1-\theta}\right)$ as $\theta + \theta^2/2$, in which case

$$y_0 = ar_0^2\theta^2/8k$$

$$\theta = \frac{1}{r_0} \sqrt{\frac{8ky_0}{a}}. \quad (32)$$

This equation is essentially the same as (4) above: by definition $\theta = (r_0^2 - r_1^2)/r_0^2$, which becomes $\theta = 2(r_0 - r_1)/r_0$ if r_1 is nearly equal to r_0 ; hence $\theta r_0 = 2(r_0 - r_1) = 2b'$, where b' is the depth of penetration, and therefore $b' = \sqrt{2ky_0/a}$, which is equation (4).

The approximate equation (32) is sufficiently accurate for most purposes up to $\theta = 0.2$. The following table can be employed over the whole range:—

Table I. Values of $u = \theta - (1 - \theta) \log_e\left(\frac{1}{1 - \theta}\right)$.

θ	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	0.95	1.0
u	0.0052	0.0215	0.0502	0.0934	0.1535	0.2335	0.3388	0.4781	0.6697	0.8002	1.0

These values are shown graphically in fig. 3.

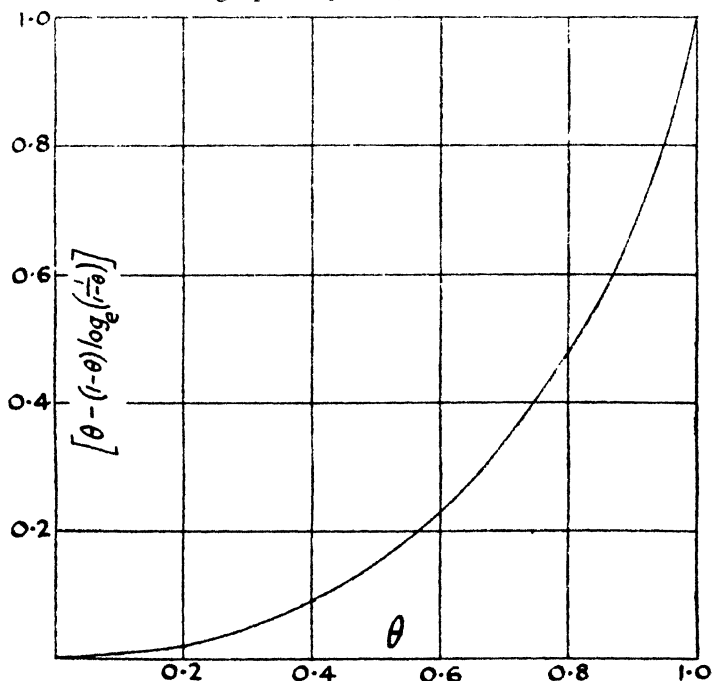


FIG. 3.—Graph of $\left[\theta - (1 - \theta) \log_e\left(\frac{1}{1 - \theta}\right)\right]$ as a function of θ . θ is the fraction of the area of a cylinder supplied with oxygen.

(a) *Absolute Values for Frog's Muscle and Nerve: the Diffusion Constant.*—No data are available for the diffusion of oxygen through nerve. It will be assumed that Krogh's value for muscle holds also for the case of nerve.

(b) (i). *Critical Diameter of a Resting Cylindrical Tissue fully supplied with Oxygen.*—Employing equation (25), $r_0' = \sqrt{4ky_0/a}$ and substituting $k = 1.4 \times 10^{-5}$, and (for muscle) $a = 7 \times 10^{-4}$ we find $r_0' = 0.283\sqrt{y_0}$.

Oxygen partial pressure, atmospheres	1	0.21	0.1	0.01	0.001	0.0001
Critical diameter, mm.	5.7	2.6	1.8	0.57	0.18	0.057

For nerve at 14° C. at rest Gerard (3) found a mean value for a of 2.7×10^{-4} c.c. per grm. per minute. Extrapolating to 20° C. by means of a temperature coefficient of 2.5 for 10° C., we obtain, for 20° C., a value of $a = 4.7 \times 10^{-4}$, so that $r_0' = 0.345\sqrt{y_0}$.

Oxygen partial pressure, atmospheres	1	0.21	0.1	0.01	0.001	0.0001
Critical diameter, mm.	6.9	3.2	2.2	0.69	0.22	0.069

Thus at 20° C. a nerve over 3 mm. thick would obtain enough oxygen at rest in air; a nerve 0.7 mm. thick (a sciatic from a frog) would obtain enough at rest in 1 per cent. oxygen; while a single fibre 7 μ thick would be adequately supplied in 0.0001 per cent. oxygen. Ordinary cylinder nitrogen may contain 0.5 per cent. oxygen: the corresponding critical diameter is 0.49 mm. Such calculations emphasise the necessity, experimentally observed, of employing exceedingly pure nitrogen if we wish to asphyxiate a small nerve.

(b) (ii). *The Fraction of the Cross-section of a Cylindrical Tissue supplied with Oxygen.*—Equation (31) and Table I, or equation (32), allow us to solve this problem. Substituting for k and a , for the case of nerve, we find

$$\theta - (1 - \theta) \log \left(\frac{1}{1 - \theta} \right) = 0.119 y_0 / r_0^2.$$

Taking the case of a frog's sciatic nerve 0.75 mm. thick ($r_0 = 0.0375$) the right-hand side becomes $85 y_0$.

Fraction of cross-section supplied with oxygen	0.05	0.1	0.2	0.4	0.7	1.0
Oxygen pressure required, atmospheres	0.000015	0.00006	0.00025	0.0011	0.0040	0.0118

We see that 5 per cent. of the whole nerve is supplied with oxygen even at a partial pressure of 0.0015 per cent. of an atmosphere. This emphasises the extreme difficulty of obtaining sufficient freedom from oxygen to secure the complete asphyxiation of a nerve. Employing the action current as an indicator of activity, and using a very sensitive galvanometer, Amberson (9) has found that the greatest care in purifying the nitrogen is necessary if activity is to be completely abolished by immersion in that gas.

In equation (31) the partial pressure of oxygen, y_0 , necessary for any given degree of asphyxiation, is proportional to the square of the thickness of the nerve. With larger nerves therefore a given fractional degree of asphyxiation is much more easily attained. For example, in the above table, the oxygen pressure required to supply a given fraction of the nerve is four times as great if the nerve be 1.5 mm. thick instead of 0.75 mm. Even so, however, extreme freedom from oxygen is necessary, with any ordinary size of nerve, if anything like the whole of it is to be completely deprived of oxygen. At a temperature lower than 20° C., where a would be largely diminished, even lower pressures of oxygen would be necessary.

Calculations for the case of muscle, analogous to those given above in § 1, (A) (b), can be made similarly if desired.

(c) *The Oxygen contained, per unit length, in a Resting Cylinder of Tissue.*—This is readily calculated, as in § 1, (A) (c), by the use of equations (26), (27) or (28). It is unnecessary to carry out further calculations of the kind here. The least pressure of oxygen inside the cylinder is on the axis; it is therefore, from equation (24), $y_0 - ar_0^2/4k$. In the case of recovery from muscular activity this quantity must be large enough, if the course of recovery is not to be interfered with by the slowness of oxygen diffusion, to supply the necessary oxygen. The conclusions from calculating with this formula will be similar to those of § 1, (A) (c) above.

(d) *The Oxygen Supply to an Active Cylinder of Tissue.*—A nerve undergoing maximal continuous activity only *doubles* its metabolism [Gerard (3)]. Thus, at 20° C., the value of a for such a state of activity is 9.4×10^{-4} instead of 4.7×10^{-4} , and the following values apply, deduced from the equation $r_0' = 0.244\sqrt{y_0}$:—

Oxygen partial pressure, atmospheres	1	0.21	0.1	0.01	0.001	0.0001
Critical diameter just supplied with oxygen, mm. ...	4.9	2.3	1.55	0.49	0.155	0.049

In calculating the oxygen pressure necessary to supply any given fraction of

Thus in a muscle 5 mm. in diameter, the size of an ordinary frog's gastrocnemius, suspended in oxygen-free Ringer, even 1 twitch per minute would lead finally to a maximum lactic acid concentration of 0.37 per cent., and an average concentration of 0.18 per cent. In a muscle 3 mm. in diameter, the size of an ordinary frog's semi-membranosus, 2 to 3 shocks per minute would lead to the fatigue maximum in the middle, while in a muscle 2 mm. in diameter the same state would be reached as the result of 5 to 7 shocks per minute. This supposes that k' is the same for high lactic acid concentrations as for low ones. Actually it is much less, so that the conditions would be worse. Practically speaking, therefore, immersing a muscle in Ringer's solution will prevent fatigue during a regular series of twitches *only if the muscle be very thin*. In a muscle only 0.1 mm. thick 5 shocks a minute regularly continued would never raise the lactic acid concentration above 0.055 per cent.

(C) *The Simultaneous Diffusion of Oxygen and Lactic Acid in the Steady State : Cylindrical Problem.*

Assume that the cylindrical tissue has a greater radius than $r_0' = \sqrt{4ky_0/a}$, so that oxygen cannot penetrate the whole, and that lactic acid is produced in the inner zone, and diffuses outwards to meet the oxygen diffusing in. The boundary conditions are exactly as in § 1, (D) above, and the solutions are

$$y = y_0 - \alpha(r_0^2 - r^2)/4k \quad (36)$$

$$y' = \alpha(r_0'^2 - r^2)/4k' - y_0 k' / k' a \quad (37)$$

Equation (36) applies, for the case of oxygen, to the outer region ; (37), for the case of lactic acid, to the inner region. The radius at which the oxygen and lactic acid meet is given by

$$r_1 = \sqrt{r_0'^2 - 4ky_0/a} = \sqrt{r_0'^2 - r_0'^2} \quad (38)$$

where r_0' is the critical radius of the cylinder which oxygen can just penetrate.

Again we see that *the distribution of the oxygen* (equation (36)) *and the distance from the axis at which the oxygen and lactic acid meet* (equation (37)) *are independent of the diffusion constant of the latter*. In pure oxygen, for a frog's muscle at 20° C. at rest, we showed above that $r_0' = 2.83$ mm. Hence for $r_0 = 3.5$ mm., $r_1 = 2.06$ mm. ; for $r_0 = 4.0$ mm., $r_1 = 2.83$ mm. ; for $r_0 = 5$ mm., $r_1 = 4.1$ mm. Thus in a muscle 10 mm. in diameter the oxygen only penetrates a zone about 1 mm. deep.

The expression for the lactic acid concentration given by equation (37)

above is identical with that of the earlier equation (33), apart from the constant $-y_0k\alpha/k'a$. Equation (33) applies to a muscle producing lactic acid when suspended in oxygen-free Ringer's solution: equation (37) shows that the effect of adding oxygen at partial pressure y_0 to the Ringer is to make a zone free of lactic acid outside the radius given by equation (38), and to lower the concentration of lactic acid throughout the inner zone by $y_0k\alpha/k'a$. This is exactly the same as for the case of a plane sheet of muscle, where we found $k\alpha/k'a=1.5 \times 10^{-3}$. Thus, as in that case, the effect of adding oxygen to the Ringer is to diminish the lactic acid in an outer zone to zero, and in an inner zone by—

0.15 per cent. (O_2 at 1 atmosphere partial pressure)

0.03 per cent. (O_2 as in air)

0.0015 per cent. (1 per cent. oxygen).

PART II.—THE KINETICS OF THE DIFFUSION OF A SINGLE SUBSTANCE.

It is often of interest in physiological problems to ascertain the time-course (a) of the penetration of a tissue of specified size and form by a substance dissolved in a fluid with which the tissue is in contact, or (b) of the outward diffusion of a substance present in, or produced by, the tissue, into a solution in which its concentration is maintained either constant or at zero. The problem may be complicated by a reaction between two diffusing substances: for example, lactic acid diffusing out of a fatigued muscle may meet oxygen diffusing in. The simple case of diffusion during a steady state has been dealt with already. In Part II we shall consider the kinetics of the diffusion of a single substance (particularly oxygen or lactic acid) and in Part III of the diffusion of two substances, which react and disappear when they meet, with special reference to the recovery process in muscle.

§ 1. DIFFUSION FROM OR INTO A PLANE SHEET.

Consider a plane sheet of muscle b cm. thick, exposed on one side to Ringer's solution or a gas, and on the other to an impermeable wall; the problem is the same as that of a plane sheet $2b$ cm. thick exposed on both sides. Let y be the concentration of oxygen at depth x cm. at time t , and k the diffusion constant. We will, for simplicity, neglect a the resting oxygen consumption of the tissue. The equation governing the flow is

$$\frac{dy}{dt} = k \frac{d^2y}{dx^2}. \quad (39)$$

Consider the case of a muscle, initially free of oxygen, suddenly exposed at time $t = 0$, and kept exposed thereafter, to a concentration $y = y_0$ at $x = 0$ and at $x = 2b$. The solution is

$$\frac{y}{y_0} = 1 - \frac{4}{\pi} \left[e^{-\frac{\lambda \pi^2 t}{4b^2}} \sin \frac{\pi x}{2b} + \frac{1}{3} e^{-\frac{9\lambda \pi^2 t}{4b^2}} \sin \frac{3\pi x}{2b} + \frac{1}{5} e^{-\frac{25\lambda \pi^2 t}{4b^2}} \sin \frac{5\pi x}{2b} + \dots \right] \quad (40)$$

In the previous discussion of the steady state of diffusion y and k were reckoned in the convenient units adopted by Krogh, employing partial pressures instead of actual concentrations. Here it is necessary to adopt absolute units of concentration. For water at 15° C. the solubility of oxygen at 1 atmosphere pressure is 0.034 c.c. per c.c. and at 20° C. it is 0.031. For muscle, according to Meyerhof and Schulz (10), the same values can be employed. Thus Krogh's diffusion constant of 1.40×10^{-5} at 20° C. becomes $1.40 \times 10^{-5} \times 1000/31$, when reckoned in c.c. of oxygen diffusing across 1 sq. cm. for a concentration gradient per cm. of 1 c.c. of O_2 per c.c. of tissue. At 20° C. therefore $k = 4.5 \times 10^{-4}$. An increase of 1 per cent. per 1° C. for Krogh's diffusion constant becomes, between 15° and 30° , one of 2.5 per cent. when reckoned in absolute units; this is due to the large change of solubility with temperature.

Now equation (40), involving an infinite series, might be difficult to handle: fortunately, however, it is very rapidly convergent: when $e^{-\frac{\lambda \pi^2 t}{4b^2}}$ is 0.7 the next term $e^{-\frac{9\lambda \pi^2 t}{4b^2}}$ is only 0.040; when the former is 0.6 the latter is 0.01. Thus, unless $e^{-\frac{\lambda \pi^2 t}{4b^2}}$ is greater than (say) 0.6, we may safely neglect all terms except the first, and write

$$\frac{y}{y_0} = 1 - \frac{4}{\pi} e^{-\frac{\lambda \pi^2 t}{4b^2}} \sin \frac{\pi x}{2b}. \quad (41)$$

Now if $e^{-\frac{\lambda \pi^2 t}{4b^2}} = 0.6$, $\frac{k\pi^2 t}{4b^2} = 0.51$ and $kt/b^2 = 0.207$. Putting $k = 4.5 \times 10^{-4}$ we find $t = 460b^2$. If b is of the order of 0.1 cm. (a thick sartorius muscle of the frog exposed on one side only) $t = 4.6$ minutes; hence, after 4.6 minutes we may safely adopt equation (41) as the solution. For $b = 0.075$ cm. (an ordinary sartorius) the corresponding value of t is 2.6 minutes.

From equation (41) we may calculate the degree of saturation y/y_0 at any point at any time. The result may be expressed as a series of curves relating y/y_0 , the degree of saturation, to x/b , the relative depth, for a series of values of $t/100b^2$. We may take $4/\pi$ to be 1.273 and (for muscle at 20° C.) $100k\pi^2/4$ to be 0.111.

Table II.

$\frac{t}{100b^2}$	5	10	15	20	25	30	40	50
$\frac{4}{\pi} e^{-\frac{k\pi^2 t}{4b^2}}$	0.731	0.420	0.242	0.139	0.080	0.046	0.015	0.005

The curves are simply sine curves of amplitudes given in the second row of the above table.

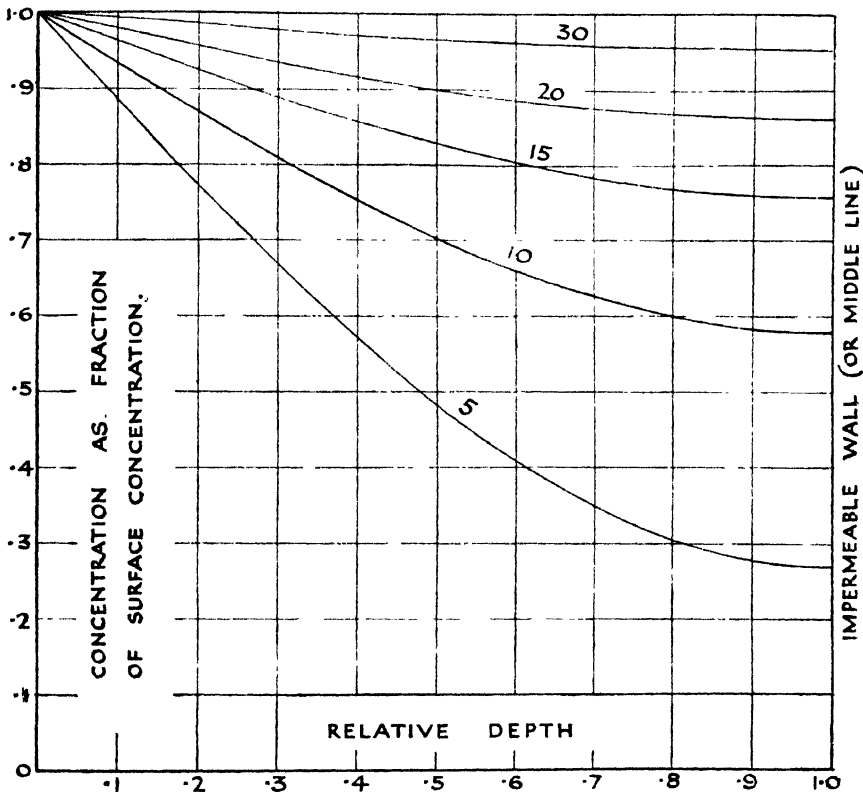


FIG. 4.—Graphs of $\frac{y}{y_0} = 1 - \frac{4}{\pi} e^{-\frac{k\pi^2 t}{4b^2}} \sin \frac{\pi x}{2b}$ as a function of x , for different values of $t/100b^2$ shown on the curves. The abscissa of this graph (x/b) represents the relative depth below the surface of the tissue, the ordinate (y/y_0) the concentration of oxygen as a fraction of that at the surface. The tissue is supposed to be of thickness $2b$, exposed on both sides, only the left half being shown; or, alternatively, to be of thickness b , exposed only on one side. Diffusion is assumed to commence at time zero, the tissue being then free of oxygen which is maintained at constant concentration y_0 at the surface. Each curve shows the distribution of oxygen inside the tissue at a given time; the different curves exhibit its progress inward as time goes on. Alternatively the curves show the passage outwards of a substance initially present at uniform concentration y_0 into a medium where the concentration is constantly zero. The diffusion constant (k) is taken as 4.5×10^{-4} . For a different diffusion constant (k') the numbers on the curves represent the values of $k't/4.5 \times 10^{-4}b^2$.

For a muscle 1 mm. thick exposed on one side, $b = 0.1$ and $t/100b^2 =$ the *time in minutes*. We see that in 20 minutes the muscle is almost completely saturated; in 5 minutes it is largely saturated. For a thin frog's sartorius $b = 0.05$ and the numbers on the curves are the times in *quarter minutes*. We see that the muscle is almost completely saturated in 5 minutes (curve 20) and more than half saturated in $1\frac{1}{4}$ minutes (curve 5). For a muscle 5 mm. thick, however, $b = 0.5$, and the numbers on the curves are marked in *25 minute* units. To attain anything like complete saturation many hours are required. We see the extraordinary effect of thickness upon the speed of attaining equilibrium by diffusion.

It should be noted that in this calculation the muscle is assumed to be exposed on one side only. If exposed on both sides, as for example in a sartorius or rectus abdominis muscle suddenly plunged into oxygenated Ringer's solution, the value of b is *half* the thickness, so that for a thin sartorius 0.5 mm. thick exposed on both sides $b = 0.025$ and the numbers on the curves are times in minutes $\div 16$; in this case nearly complete saturation with oxygen is attained within a minute.

The total oxygen inside the muscle at time t bears to the full saturation amount a ratio

$$\frac{\int_0^b y dx}{y_0 b} = 1 - \frac{8}{\pi} \left[e^{-\frac{k\pi^2 t}{4b^2}} + \frac{1}{9} e^{-\frac{9k\pi^2 t}{4b^2}} + \frac{1}{25} e^{-\frac{25k\pi^2 t}{4b^2}} + \dots \right]. \quad (42)$$

This represents the average degree of saturation at time t . The series is rapidly convergent when t is large, but it is convenient to be able to use it for quite short times, so that in the following calculation all the necessary terms of the series have been taken into account, to make it correct to three places of decimals. It is calculated for a muscle at 20°C. ($k = 4.5 \times 10^{-4}$).

Table III.

$\frac{t}{100b^2}$	0.1	0.2	0.3	0.5	0.7	1.0	2	3	5
Average saturation . .	0.075	0.105	0.130	0.169	0.200	0.239	0.332	0.414	0.534
$\frac{t}{100b^2}$	7	10	15	20	25	30	40	50	
Average saturation . . .	0.628	0.732	0.846	0.911	0.949	0.971	0.990	0.997	

For a muscle 1 mm. thick exposed on one side only, or 2 mm. thick exposed on both sides, the first row represents time in minutes, and the muscle is seen to be 73 per cent. saturated in 10 minutes; for a muscle $\frac{1}{2}$ mm. thick exposed on one side, or 1 mm. thick exposed on both sides, the first row represents time expressed in $\frac{1}{4}$ minutes and we see that the muscle is 99 per cent. saturated in 10 minutes, 73 per cent. saturated in $2\frac{1}{2}$ minutes.

It should be noted that the calculation given here applies equally to the case of oxygen diffusing out or of oxygen diffusing in. If the muscle be in oxygen and this be suddenly replaced by nitrogen the oxygen immediately starts to diffuse out, and the course of its movement out follows the series of curves shown in fig. 4 but reversed. Indeed the calculations given here apply equally to *any* sudden change in the external concentration; if y_0 be suddenly altered to y_1 the concentration proceeds to change and fig. 4 gives the value of $\frac{y - y_0}{y_1 - y_0}$ as a function of x and t . The change from any pre-existing conditions follows the course here calculated.

The oxygen dissolved in a muscle at 1 atmosphere pressure at 20°C ., neglecting metabolism, is about 0.031 c.c. per c.c. This would give about 0.155 calorie, or enough for 22 maximal twitches. If a muscle previously in oxygen be suddenly placed in nitrogen Table III gives the average "unsaturation" at the times in the first row. A muscle 0.7 mm. thick exposed on one side will in 10 minutes, ($\frac{t}{100b^2} = 20$), even neglecting its metabolism, be 91 per cent. unsaturated, i.e., only 9 per cent. of its original oxygen will be left. This is enough only for two twitches. Thus 10 minutes in nitrogen with 2 twitches will completely exhaust all the oxygen previously dissolved in it.

It may, on occasion, be convenient to have data similar to that of Table III available for any value of k . The average saturation is a function simply of kt/b^2 , and in fig. 5 this relation is shown by the curve denoted kt/b^2 (plane). Let us consider how long it will take for 50 per cent. and for 90 per cent. of the lactic acid in a plane sheet of muscle 0.75 mm. thick exposed on both sides to diffuse out into surrounding Ringer's solution: $k = 6 \times 10^{-5}$, $b = 0.0375$. For 50 per cent., from fig. 5, $kt/b^2 = 0.2$, so that $t = 4.7$ minutes. For 90 per cent., $kt/b^2 = 0.84$, so that $t = 19.3$ minutes. Let us consider the same problem for a muscle four times as thick, 3 mm.: the times are 16 times as great, for 50 per cent. to come out 75 minutes, for 90 per cent. 309 minutes. We see again the extraordinary differences between thick and thin tissues, in relation to the speed of diffusion.

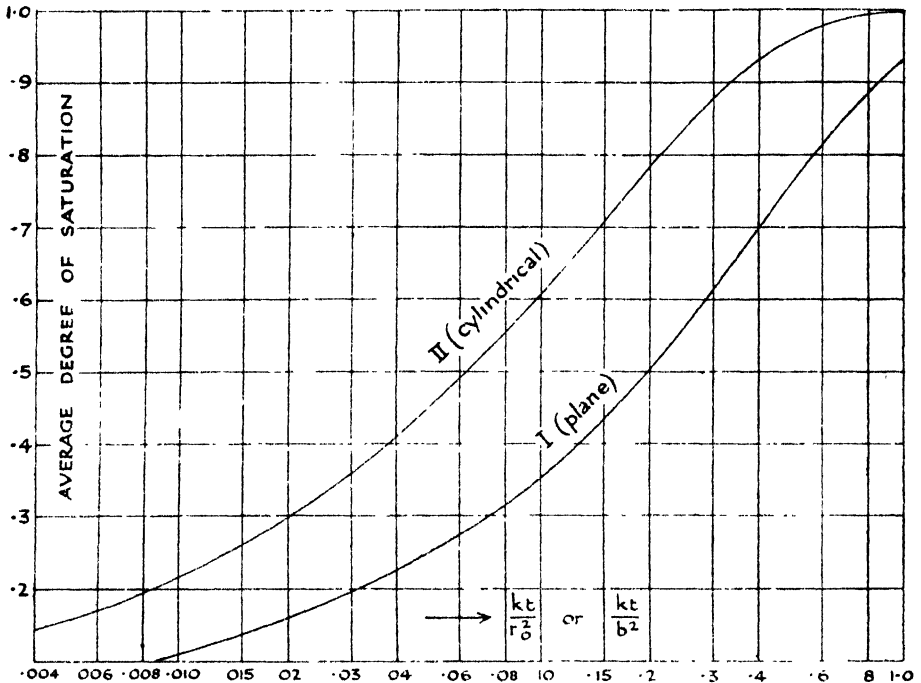


FIG. 5.—Graphs showing the average degree of saturation of a tissue, at various times after exposure to a constant concentration at the surface. Two curves are shown, I for diffusion into a plane sheet of thickness $2b$ exposed on both sides (or of thickness b exposed on one side), and II for diffusion into a cylinder of radius r_0 . k is the diffusion constant and t is the time, kt/b^2 and kt/r_0^2 being the abscissæ on a logarithmic scale for I and II respectively. Note that these curves are available for any size of tissue and any diffusion constant. See equations (42) and (47).

§ 2. DIFFUSION FROM OR INTO A CYLINDER.

The diffusion equation, neglecting the metabolism of the tissue, is

$$\frac{dy}{dt} = \frac{k}{r} \frac{d}{dr} \left(r \frac{dy}{dr} \right). \quad (43)$$

Put $y = ue^{-\lambda t}$, then the equation becomes

$$\frac{d^2u}{d \left(r \sqrt{\frac{\lambda}{k}} \right)^2} + \frac{1}{r \sqrt{\frac{\lambda}{k}}} \frac{du}{d \left(r \sqrt{\frac{\lambda}{k}} \right)} + u = 0.$$

The solution of this equation is

$$u = \Sigma \left[AJ_0 \left(r \sqrt{\frac{\lambda}{k}} \right) + BY_0 \left(r \sqrt{\frac{\lambda}{k}} \right) \right] + \text{a constant},$$

where J_0 and Y_0 are the Bessel's functions of zero order.

Now $Y_0(x)$ is infinite at $x = 0$, hence if the region under consideration includes the axis all the B 's must be zero, and we are left with

$$y = \Sigma A e^{-\lambda^2 t} J_0 \left(r \sqrt{\frac{\lambda}{k}} \right) + \text{a constant.} \quad (44)$$

Now consider the cylinder, previously oxygen-free, to be suddenly introduced into oxygen at concentration y_0 . We have :—

$$\text{At } r = r_0, \quad y = y_0 \text{ for all times ;}$$

$$\text{At } t = 0, \quad y = 0 \text{ for all } r\text{'s.}$$

The solution can be shown to be

$$\frac{y}{y_0} = 1 - 2 \left[\frac{1}{v_1 J_1(v_1)} e^{-\frac{k v_1^2 t}{r_0^2}} J_0 \left(\frac{r}{r_0} v_1 \right) + \frac{1}{v_2 J_1(v_2)} e^{-\frac{k v_2^2 t}{r_0^2}} J_0 \left(\frac{r}{r_0} v_2 \right) + \dots \right] \quad (45)$$

where $v_1, v_2, v_3, v_4, v_5, \dots$ are the zeros of $J_0(x)$ and have the values 2.405, 5.520, 8.654, 11.79, 14.93, ... while $J_1(v_1), J_1(v_2), J_1(v_3), \dots$ have the values $-0.5191, +0.3103, -0.2714, +0.2325, -0.2066, \dots$

The value of y/y_0 on the axis is

$$\left| \frac{y}{y_0} \right|_{r=0} = 1 - 2 \left[\frac{e^{-\frac{k v_1^2 t}{r_0^2}}}{v_1 J_1(v_1)} + \frac{e^{-\frac{k v_2^2 t}{r_0^2}}}{v_2 J_1(v_2)} + \dots \right] \quad (46)$$

and the average saturation inside the cylinder at time t is

$$\frac{\bar{y}}{y_0} = 1 - 4 \left[\frac{e^{-\frac{k v_1^2 t}{r_0^2}}}{v_1^2} + \frac{e^{-\frac{k v_2^2 t}{r_0^2}}}{v_2^2} + \dots \right]. \quad (47)$$

This series is not very rapidly convergent for small values of t : it is necessary therefore to consider several terms. The result of evaluating equation (47) can be expressed as a function of kt/r_0^2 as in the following table :—

Table IV.

kt/r_0^2	0.003	0.005	0.007	0.010	0.02	0.03	0.04	0.05	0.07
\bar{y}/y_0	0.13	0.159	0.183	0.216	0.299	0.361	0.410	0.453	0.524
kt/r_0^2	0.10	0.15	0.20	0.3	0.4	0.5	0.7	1.0	
\bar{y}/y_0	0.606	0.708	0.782	0.878	0.932	0.961	0.988	0.998	

These numbers are plotted in fig. 5 in the curve denoted kt/r_0^2 (cylindrical). Some interesting examples may be taken. A frog's sciatic nerve 0.7 mm.

thick ($r_0 = 0.035$) is suddenly exposed to oxygen. According to fig. 5 it is 90 per cent. saturated when $kt/r_0^2 = 0.333$, 50 per cent. when $kt/r_0^2 = 0.063$. Putting $k = 4.5 \times 10^{-4}$, $r_0 = 0.035$, we find $t = 0.9$ minute for 90 per cent. saturation, only 0.17 minute (about 10 seconds!) for 50 per cent. saturation. Conversely if a nerve of the same diameter previously in oxygen be placed suddenly in nitrogen its oxygen diffuses out and its "unsaturation" in 0.9 minute is 90 per cent., in 2.2 minutes 99 per cent.; in other words 99 per cent. of its previously contained oxygen has diffused out within 2.2 minutes.

This affords a very pertinent comment on the experimental fact that a nerve of this size may continue to show an action current on stimulation, after exposure to the purest nitrogen for 2 hours; it is clear that nerve has some "accumulator mechanism" as well as muscle. The oxygen dissolved inside such a nerve in air is, at 20° C., about 0.006 c.c. per c.c. After replacing the air by pure nitrogen, within 2.2 minutes there will be only 0.00006 c.c. of O_2 per c.c. remaining, even neglecting metabolism. The rate of oxygen consumption of resting nerve at 20° C. is about 0.0005 c.c. per c.c. per minute; thus at the end of 2.2 minutes there would be only 5 seconds supply of oxygen left dissolved inside the nerve. It is clear that in really pure nitrogen a nerve of this small size rapidly becomes entirely free of molecular oxygen; and yet it can continue to function for long periods.

If we take the case of tissues of greater diameter we find a very different state of affairs. How long, for example, is required for oxygen to penetrate a tissue 1 cm. in diameter ($r_0 = 0.5$)? From fig. 5, 90 per cent. saturation is reached when $kt/r_0^2 = 0.333$, so that $t = 185$ minutes; even 50 per cent. saturation requires 35 minutes. These times are so long, and the cylinder is so thick, that the metabolism of the tissue itself during the diffusion process cannot be neglected. In most nerves, however, as distinguished from muscles, the diameters are so small that a considerable degree of saturation is attained in a very few minutes. Even with a large nerve of 1.5 mm. diameter ($r_0 = 0.075$) 50 per cent. saturation is attained when (see fig. 5) $kt/r_0^2 = 0.063$, i.e., when $t = 0.78$ minute. We see again how effectively a nerve of ordinary size can be supplied with oxygen by diffusion alone: an intimate blood supply is unnecessary.

Just as in the case of plane diffusion the curve of fig. 5 can be employed with any diffusion constant. A single muscle fibre 50 μ in diameter, $r_0 = 0.0025$ cm., contracts, producing lactic acid, which immediately proceeds to diffuse into the surrounding medium. How long does it take for 90 per cent. of this

to diffuse out? We will assume for the fibre a diffusion constant of 5×10^{-6} (see Eggleton, Eggleton and Hill (7)). From fig. 5 for 90 per cent. $kt/r_0^2 = 0.333$. Hence $t = 0.41$ minute = about 25 seconds: 50 per cent. of it can be calculated to pass out in 0.078 minute, about 5 seconds. This emphasises again the quickness of diffusion in the case of systems of small size, even with a very low coefficient of diffusion. A muscle 8 mm. thick (a large frog's gastrocnemius) shows a very different picture. Immersed in Ringer's solution, even with the higher diffusion constant of muscle in bulk, viz., 6×10^{-5} , it would take 890 minutes to lose 90 per cent. of its lactic acid, 168 minutes to lose one half. With the lower diffusion constant found for fatigued muscle the process would occupy days.

(a) *The "Permeability" of Tissues.*—Various claims have, from time to time, been made to have established a change in the "permeability" of muscle under various conditions. It is difficult to imagine that, for substances such as phosphate and lactate, diffusion through the lymph interspaces of the tissue does not exert a considerable influence on the observed results. The values of the diffusion constants found experimentally are so much lower than those of the same substances in free solution (see Stella (11), Eggleton, Eggleton and Hill (7)) that if only 5 per cent. of the whole tissue were free lymph, diffusion through the latter would exert a preponderant effect. If the diffusion constant through lymph were 6×10^{-1} , and through muscle fibre 5×10^{-6} changes (unless of extreme magnitude) in the "permeability" of (i.e., presumably in the diffusion constant through) the fibre would exert a very small effect; it would be much more natural to attribute observed changes in the speed of diffusion to alterations in the amount of free lymph in the interspaces between the fibres. Given an amount of lymph occupying 5 to 10 per cent. of the volume of the tissue, even an extremely small diffusion constant through living fibres would exert only an insignificant effect in diminishing the speed of diffusion through a mass of tissue. In a muscle fibre 50 μ thick ($r_0 = 0.0025$), diffusion equilibrium between the inside and the outside will be reached in a time which can be roughly calculated by the aid of fig. 5. For 20 per cent. of the dissolved substance to come out kt/r_0^2 is required to be about 0.0084, $kt = 5 \times 10^{-8}$ approximately. Thus if k were only one-thousandth of the value obtaining in free solution (say 5×10^{-7}), t would still be only 0.1 minute = 6 seconds. Thus, in spite of the low diffusion constant, the passage from inside the fibre to the lymph is still so rapid as to provide only an insignificant delay in diffusion through the latter. This rapidity is due to the extremely small dimensions of the living cell. The "permeability" of,

i.e., the diffusion constant through, the living fibre will affect the observed rate of diffusion under certain conditions only :—

- (a) If it be very large, of the same order of size as in free solution ; this is the case with oxygen.
- (b) If it be extremely small. say, one-millionth of that in free solution. in which case the time to diffuse out from a single fibre will be so long as seriously to delay the passage along the lymph-spaces.
- (c) If the lymph-spaces be abolished by a rise of osmotic pressure in the fibres.

Under other conditions alterations in the observed rate of diffusion tell us more about the osmotic pressure inside the fibres, and of changes in the size of the lymph-spaces, than of alterations in the “ permeability ” of the muscle.

§ 3. DIFFUSION FROM OR INTO A “ SEMI-INFINITE ” SOLID BOUNDED BY A PLANE SURFACE.

The equations derived for this case have been employed by Eggleton, Eggleton and Hill (7) and by Stella (11) for the determination of the diffusion constants of phosphate and lactate through muscle. If a semi-infinite solid extending from $x = 0$ to $x = \infty$ be suddenly brought into contact with a large quantity of well-stirred liquid containing a diffusible substance in concentration y_0 , then the substance will immediately begin to diffuse into the solid, at first rapidly, but later more slowly as the surface layers are saturated with the substance in question. The *rate* of diffusion inwards at any time t from the beginning, reckoned per unit of surface, is

$$\text{Rate} = y_0 \sqrt{k/\pi t}, \quad (48)$$

while the total *amount* which has diffused across unit area of the boundary up to time t is

$$\text{Amount} = 2y_0 \sqrt{kt/\pi}. \quad (49)$$

These formulæ are very convenient for determining the diffusion constant of a substance into, or out from, a tissue of somewhat irregular shape, where no exact mathematical treatment is possible. Provided that the time t of diffusion is short enough, any body of not too irregular shape can be regarded as a semi-infinite solid ; the only condition is that the layer affected by diffusion within time t should be so thin that the concentration is unaltered in the interior of the solid. So long as the amount diffusing in, or out, is accurately proportional to the square root of the time (and this can be examined experimentally),

equation (49) is obeyed and the constant of the proportion is $2y_0 \sqrt{k/\pi}$, from which k can be calculated. Details of the method employed in determining the diffusion constants of phosphate and lactic acid in this way have been given by Stella (11) and by Eggleton, Eggleton and Hill (7).

It is obvious that with a body of ordinary size the equations cannot hold indefinitely as t increases. The amount diffusing must become a smaller and smaller multiple of \sqrt{t} as time goes on, since with $t = \infty$ the amount is finite. It is desirable to know approximately how long, for a body of given size and shape, diffusion may be allowed to proceed, without serious deviation from the formulæ. A case may be taken which has been accurately calculated in § 2 above, that of a cylinder of radius r_0 suddenly immersed in a solution. Referring to Table IV, at a time given by $kt/r_0^2 = 0.005$ the quantity which has diffused, per unit length, is 0.159 of the maximum possible at $t = \infty$, in amount therefore $0.159\pi r_0^2 y_0$. Treating the cylinder as a semi-infinite solid, of surface $2\pi r_0$ per unit length, the amount diffusing, according to equation (49), is $2\pi r_0 \cdot 2y_0 \sqrt{k t/\pi} = 4r_0 y_0 \sqrt{\pi k t}$. The ratio of this quantity to $0.159\pi r_0^2 y_0$ is $4 \sqrt{k t}/(0.159 r_0 \sqrt{\pi})$. Substituting $\sqrt{k t}/r_0 = \sqrt{0.005} = 0.0707$ the ratio becomes 1.001. Thus for $kt/r_0^2 = 0.005$ the approximation of equation (49) is quite sufficient. For $kt/r_0^2 = 0.007$ the result of equation (49) is 3 per cent. too great; for $kt/r_0^2 = 0.01$, $4\frac{1}{2}$ per cent. too great. In this last case diffusion has proceeded to 0.216 of its full extent, so that for reasonably accurate results (in such experiments as these) we may safely employ times of diffusion long enough to allow diffusion to proceed to 15 or 20 per cent. completion. For a cylinder of 1.2 cm. diameter ($r_0 = 0.6$), which is about the size of a frog's leg, and with a diffusion constant of 6×10^{-5} , and a time of 30 minutes (the greater times in the experiments of Eggleton, Eggleton and Hill were usually about 30 minutes) we have $kt/r_0^2 = 0.005$, a value for which we have already shown that the error in applying the formula is only 0.4 per cent.

PART III.—THE KINETICS OF THE DIFFUSION OF TWO REACTING SUBSTANCES.

The rate of diffusion of oxygen into a fatigued muscle is affected by the fact that it is used up in the oxidative removal of lactic acid. When oxygen is suddenly introduced around such a muscle the process of recovery begins at once, as shown by the immediate appearance of recovery heat ((8), p. 188). The oxygen slowly penetrates the tissue, until finally the whole has recovered. In a general way we may think of a recovered "oxygen" zone and a fatigued

"lactic acid" zone, and imagine that the boundary between these slowly advances inwards as recovery proceeds. The process of oxidation is not, of course, instantaneous, nor is the boundary strictly a thin surface, but rather a layer of finite thickness lying between the recovered and the fatigued zones. For simplicity, however, in the mathematical treatment, we will imagine that the oxygen and the lactic acid react together in an indefinitely thin layer, which slowly sweeps forward through the tissue until the whole has recovered.

The diffusion of lactic acid is not so rapid as that of oxygen, so that, as an approximation we will first assume (Case I) that oxygen alone diffuses, meeting lactic acid as it advances. Then, however, we will deal with the more difficult problem (Case II) of oxygen and lactic acid both diffusing. For the steady state, Case II has been considered already in Part I.

§ 1. THE DIFFUSION OF OXYGEN INTO A PLANE SHEET OF MUSCLE IN WHICH RECOVERY IS NECESSARY AT EVERY POINT BEFORE THE OXYGEN CAN ADVANCE.

CASE I.—OXYGEN ALONE DIFFUSING.

The diffusion equation for oxygen as before is

$$\frac{dy}{dt} = k \frac{d^2y}{dx^2}.$$

The boundary conditions are :—

- (i) At $x = 0$, $y = y_0$ always (*i.e.*, the oxygen is maintained at a constant concentration at the surface).
- (ii) At $t = 0$ there is an "oxygen debt" λ per c.c. of tissue everywhere.
- (iii) At $t = \infty$, $y = y_0$ everywhere (*i.e.*, recovery is finally complete).
- (iv) At $x = \xi$, the point where $y = 0$, *i.e.*, where recovery is, at the moment, proceeding, the rate at which oxygen is diffusing onwards is equal to the rate at which the "oxygen debt" is being paid off as the front advances. The rate of diffusion at this point is $k(-dy/dx)$; the speed with which the front advances is $d\xi/dt$, so that the rate at which the oxygen debt is being paid off is $\lambda d\xi/dt$. Hence

$$-k \left(\frac{dy}{dx} \right)_{x=\xi} = \lambda \frac{d\xi}{dt}. \quad (50)$$

The solution for the region containing oxygen is

$$y = y_0 - A \int_0^x e^{-\theta^2} d\theta, \quad (51)$$

where $z = x/2\sqrt{kt}$ and A is a constant to be determined. This can be shown to satisfy the differential equation, and conditions (i) and (iii).

Where $y = 0$,

$$y_0 = \frac{A\sqrt{\pi}}{2} \left[\frac{2}{\sqrt{\pi}} \int_0^{\infty} e^{-\theta^2} d\theta \right]$$

(the $2/\sqrt{\pi}$ is introduced inside the bracket, since $2/\sqrt{\pi} \int_0^{\infty} e^{-\theta^2} d\theta$ is the "probability integral" which is tabulated, and has the value unity when $z = \infty$).

If we know A this equation for z can be solved numerically by tables (or a graph) of the function on the right. Let the solution be $z = \zeta$. Then the distance ξ to which recovery has advanced in time t is given (since $z = x/2\sqrt{kt}$) by

$$\xi = 2\zeta\sqrt{kt}. \quad (52)$$

Hence $d\xi/dt = \zeta\sqrt{k/t}$, and

$$-k(dy/dx)_{x=\xi} = -k(dy/dz)(dz/dx)_{x=\xi} = kAe^{-\zeta^2} 2\sqrt{kt}.$$

Hence boundary condition (iv) gives us

$$A = 2\zeta\lambda e^{\zeta^2}$$

and the equation to find ζ can be written

$$y_0 = \lambda\sqrt{\pi}\zeta e^{\zeta^2} \left[\frac{2}{\sqrt{\pi}} \int_0^{\zeta} e^{-\theta^2} d\theta \right]. \quad (53)$$

The amount V of oxygen used, per sq. cm. of diffusing surface, up to time t , is $\lambda\xi$, which may be written

$$V = 2\lambda\zeta\sqrt{kt}. \quad (54)$$

It is necessary to tabulate the values of the function

$$u = \frac{\sqrt{\pi}}{2} \zeta e^{\zeta^2} \left[\frac{2}{\sqrt{\pi}} \int_0^{\zeta} e^{-\theta^2} d\theta \right].$$

We then write $u = y_0/2\lambda$, and looking up this value of u in the table we find the corresponding value of ζ . The function u occurs again below (Case II) and in fig. 6 it is shown on a logarithmic scale.

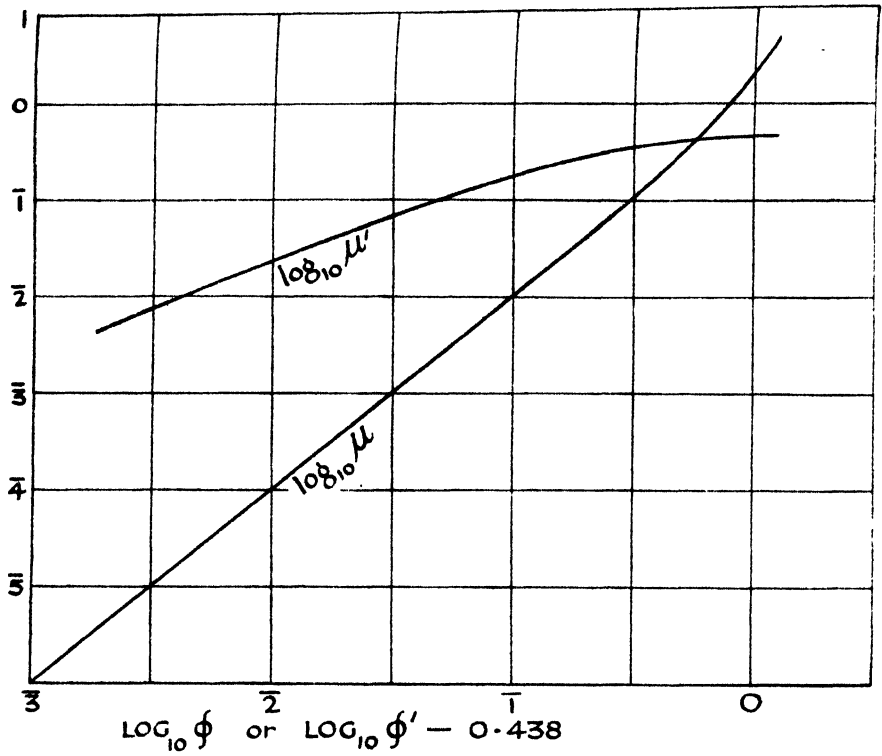


FIG. 6.—Graphs of the quantities $u = \phi e^{\phi^2} \int_0^\phi e^{-\theta^2} d\theta$ and $u' = \phi' e^{\phi'^2} \int_{\phi'}^\infty e^{-\theta^2} d\theta$, as functions of ϕ and $\phi'/2.74$ respectively. These are shown on a logarithmic scale, $\log_{10} u$ as a function of $\log_{10} \phi$, $\log_{10} u'$ as a function of $\log_{10} \phi' - 0.438$. For the use of these graphs see pp. 80 and 83.

Table V.

ζ	0.001	0.002	0.005	0.010	0.02	0.05	0.10	0.2	0.3
u	0.000001	0.000004	0.000025	0.0001	0.0004	0.0025	0.0101	0.0412	0.0955
ζ	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.2	
u	0.178	0.296	0.46	0.685	1.00	1.43	2.03	4.07	

When the oxygen front advances only slowly, *i.e.*, when y_0 is small or λ large, the quantity on the right of equation (53) can be greatly simplified, since ζ will be small enough to allow us to neglect all but its lowest powers. We find in this case that $u = \zeta^2$. It is clear from an inspection of Table V

that up to $\zeta = 0.2$, where $u = 0.0412$ as correctly calculated and $u = 0.04$ from the simple formula, no error of importance results from assuming the latter. Even at $\zeta = 0.4$ the error involved in adopting the simpler formula is only 10 per cent. Over a comparatively wide range therefore we may write

$$y_0/\lambda = 2\zeta^2 = \xi^2/2kt \quad \text{or} \quad \xi^2/t = 2ky_0/\lambda. \quad (55)$$

This simple formula, which is accurate enough for most purposes and can be applied as a first approximation to a tissue of any shape and size, gives the depth ξ to which the recovery front has penetrated in time t , for any partial pressure y_0 of oxygen and any oxygen debt λ .

The amount of oxygen used, up to time t , per sq. cm. of diffusing surface was shown in equation (54) to be $2\lambda\zeta\sqrt{kt}$. With the simplified formula it is $\xi\lambda = \sqrt{2ky_0\lambda t}$, hence, approximately,

$$V = \sqrt{2ky_0\lambda t}. \quad (56)$$

The quantity of oxygen used is proportional to the square root of the time, of the oxygen debt and of the oxygen concentration. By measuring the oxygen consumption of a fatigued muscle, as a function of the time, it should be possible to verify equation (56) and to determine the constant.

It is possible to deduce the simplified formulæ (55) and (56) very easily. Imagine the oxygen front to advance so slowly that a steady state is set up behind it. We have

$$dy/dt = 0 = k d^2y/dx^2.$$

Hence

$$y = y_0 (1 - x/\xi).$$

Now at the advancing surface, as before

$$\begin{aligned} \lambda d\xi/dt &= -k (dy/dx)_{x=\xi} \\ &= ky_0/\xi. \end{aligned}$$

Thus

$$\xi d\xi/dt = ky_0/\lambda$$

and

$$\xi^2/t = 2ky_0/\lambda,$$

which is equation (55).

The same method can be employed to take account also of the metabolism of the tissue while recovery is going on. Imagine that in the oxygen region there is an oxygen consumption at the rate of a c.c. per c.c. per minute, while in the lactic acid region the "oxygen debt" is increasing at the rate of a c.c. per minute. Then the equation for the steady state in the oxygen region is (2) above, viz., $a = k d^2y/dx^2$, so that $y = ax^2/2k + Bx + y_0$.

Since $y = 0$ at $x = \xi$ we have

$$B = -a\xi/2k - y_0/\xi.$$

At the oxygen front

$$\lambda d\xi/dt = -k(dy/dx)_{x=\xi} = ky_0/\xi - a\xi/2.$$

But

$$\lambda = \lambda_0 + at, \quad \text{so that} \quad \frac{-a\xi d\xi}{ky_0 - a\xi^2/2} = \frac{-a dt}{\lambda_0 + at},$$

which on integration becomes

$$1 - a\xi^2/2y_0k = 1/(1 + at/\lambda_0).$$

This equation simplifies to

$$\frac{\xi^2}{t} = \frac{2ky_0}{\lambda_0 + at}, \quad (57)$$

which should be compared with (55). The total amount of oxygen used in time t is as before $\xi(\lambda_0 + at)$ which becomes

$$V = \sqrt{2ky_0(\lambda_0 + at)}t, \quad (58)$$

which should be compared with (56).

Numerical Examples. (1) *Equation (53).* For muscle at 20° C. $k = 4.5 \times 10^{-4}$, $2\sqrt{k} = 4.24 \times 10^{-2}$, y_0 for 1 atmosphere of oxygen = 0.031 c.c. per c.c. Assume that there is 0.1 per cent. of lactic acid present, which with an "oxidative quotient" of 4.8 is equivalent to an oxygen debt of 0.155 c.c. per c.c. Then $u = y_0/2\lambda = 0.031/0.310 = 0.1$. Hence from fig. 6, $\zeta = 0.306$, and $\xi = 0.013\sqrt{t}$. Thus in 10 minutes the recovery front will have advanced 0.41 mm., in 40 minutes 0.82 mm. Thus it should take about 40 minutes in oxygen to complete the recovery of a muscle containing 0.1 per cent. of lactic acid and 0.82 mm. thick, exposed on one side only. This corresponds to the case of a partially fatigued frog's sartorius placed on a thermopile, where we know (8), p. 188) that the recovery heat takes about this time to attain its full value.

For air, instead of oxygen, $y_0 = 0.0065$ and $y_0/2\lambda = 0.021$. Hence $\zeta = 0.14$ and $\xi = 0.0059\sqrt{t}$. If $\xi = 0.41$ mm., $t = 48$ minutes (instead of 10 for oxygen); if $\xi = 0.82$ mm., $t = 193$ minutes (instead of 40).

(2) *Simplified Equation (55).* (a) Putting $\lambda = 0.155$, $y_0 = 0.031$, $k = 4.5 \times 10^{-4}$, we find $\xi^2/t = 1.8 \times 10^{-4}$. If $t = 10$, $\xi = 0.042$ cm. = 0.42 mm., practically the same as the 0.41 mm. just calculated by equation (53).

(b) For the same case equation (56) gives us $V = \sqrt{2ky_0\lambda t} = 0.00208\sqrt{t}$ c.c. per sq. cm. of surface. This is a quantity which should be easily measurable experimentally.

(3) *Equation (57).*—For resting muscle at 20° C., $a = 7 \times 10^{-4}$ and for 0.1 per cent. lactic acid $\lambda_0 = 0.155$. Hence the term $at/\lambda_0 = 4.5 \times 10^{-3}t$. For $t = 10$ minutes, the case discussed above, ξ^2/t is $0.957 \times 2ky_0/\lambda_0$, instead of $2ky_0/\lambda_0$ which is the value if we neglect the resting metabolism. For $t = 40$ minutes, $at/\lambda_0 = 0.18$ and $\xi^2/t = 0.847 \times 2ky_0/\lambda_0$. Thus ξ is diminished by the metabolism by only 2.2 and 8 per cent. respectively in these two cases. If, however, the oxygen debt be less the effect of the metabolism is relatively greater. For $\lambda_0 = 0.0155$ (0.01 per cent. lactic acid), $at/\lambda_0 = 4.5 \times 10^{-2}t$, and ξ^2/t becomes $0.69 \times 2ky_0/\lambda_0$ and $0.357 \times 2ky_0/\lambda_0$ for $t = 10$ and $t = 40$ minutes, so that ξ is diminished by 17 and by 40 per cent. respectively by the metabolism.

Similarly, with the aid of equation (58), the resting metabolism during recovery can be shown to exert a comparatively small influence on the total oxygen used, unless either the oxygen debt be small or the time allowed very great.

CASE II.—LACTIC ACID DIFFUSING AS WELL AS OXYGEN.

(a) *Sheet of Infinite Thickness.*—For simplicity we will assume that there is no resting metabolism, an assumption involving no considerable error for times that are not too great. The oxygen is supposed to be maintained at constant concentration y_0 at the surface $x = 0$; the lactic acid to be initially at concentration y'_0 from $x = 0$ to $x = \infty$. Let y and k refer to oxygen, y' and k' to lactic acid. We require to know how rapidly the boundary between the "oxygen" zone and the "lactic acid" zone progresses inwards, and how much oxygen is used in a given time.

The differential equations are :—

$$dy/dt = k d^2y/dx^2 \quad \text{and} \quad dy'/dt = k' d^2y'/dx^2.$$

The boundary conditions are :—

- (1) At $x = 0$, $y = y_0$ always.
- (2) At $x = \infty$, $y' = y'_0$ always.
- (3) At $t = 0$, $y' = y'_0$ everywhere.
- (4) At $t = \infty$, $y = y_0$ everywhere.

(5) At the boundary $x = \xi$, where $y = y' = 0$, and recovery is proceeding at the moment, the amount of oxygen which diffuses forward in unit time $k(-dy/dx)$ is exactly sufficient to complete the oxidative removal of the lactic acid which diffuses backward in the same time, $k'dy'/dx$. Therefore

$$\frac{-k dy/dx}{k' dy'/dx} = \mu, \tag{58}$$

where μ is the number of c.c. of oxygen required to effect the oxidative removal of 1 gram of lactic acid.

As in Case I the following can be shown to satisfy the differential equations, and boundary conditions (1) to (4).

$$\left. \begin{aligned} y &= y_0 - A \int_0^{z/2\sqrt{k}} e^{-\theta^2} d\theta \\ y' &= y'_0 - A' \int_{z/2\sqrt{k'}}^{\infty} e^{-\theta^2} d\theta \end{aligned} \right\}, \quad (59)$$

where $z = x/\sqrt{t}$ and A and A' are constants to be determined.

For the boundary $x = \xi$ between the recovered and the fatigued zones, where $y = y' = 0$, we have a particular value of z , $z = \zeta$, for which

$$\left. \begin{aligned} y_0 &= A \int_0^{\zeta/2\sqrt{k}} e^{-\theta^2} d\theta \\ y'_0 &= A' \int_{\zeta/2\sqrt{k'}}^{\infty} e^{-\theta^2} d\theta \end{aligned} \right\}, \quad (60)$$

and boundary condition (5) may be written

$$\mu = \left[\frac{-k dy/dx}{k' dy'/dx} \right]_{x=\xi} = \frac{\frac{Ak}{\sqrt{kt}} e^{-\zeta^2/4k}}{\frac{A'k'}{\sqrt{k't}} e^{-\zeta^2/4k'}}.$$

Eliminating A and A' from these three equations and rearranging we find,

$$\frac{\mu y'_0}{y_0} = \frac{\frac{e^{\zeta^2/4k'}}{\sqrt{k'}} \int_{\zeta/2\sqrt{k'}}^{\infty} e^{-\theta^2} d\theta}{\frac{e^{\zeta^2/4k}}{\sqrt{k}} \int_0^{\zeta/2\sqrt{k}} e^{-\theta^2} d\theta}. \quad (61)$$

Let

$$\zeta/2\sqrt{k} = \phi, \quad \zeta/2\sqrt{k'} = \phi', \quad u = \phi e^{\phi^2} \int_0^{\phi} e^{-\theta^2} d\theta$$

and

$$u' = \phi' e^{\phi'^2} \int_{\phi'}^{\infty} e^{-\theta^2} d\theta = \phi' e^{\phi'^2} \left(\frac{\sqrt{\pi}}{2} - \int_0^{\phi'} e^{-\theta^2} d\theta \right).$$

Then from equation (61) above, multiplying numerator and denominator by ζ , we have

$$\frac{\mu y'_0}{y_0} = \frac{\frac{\zeta}{2\sqrt{k'}} e^{\zeta^2/4k'} \int_{\zeta/2\sqrt{k'}}^{\infty} e^{-\theta^2} d\theta}{\frac{\zeta}{2\sqrt{k}} e^{\zeta^2/4k} \int_0^{\zeta/2\sqrt{k}} e^{-\theta^2} d\theta} = \frac{u'}{u}.$$

Now $\mu y_0' = \lambda$, the oxygen debt per c.c. Hence $\lambda/y_0 = u'/u$. This is most conveniently expressed in logarithmic form

$$\log_{10} u - \log_{10} u' = \log_{10} y_0 - \log_{10} \lambda \quad (62)$$

The values of $\log_{10} u$ and $\log_{10} u'$, as functions of ϕ and ϕ' , are given in the following table: --

Table VI. -Values of $u = \phi e^{\phi^2} \int_0^{\phi} e^{-\theta^2} d\theta$ and of $u' = \phi' e^{\phi'^2} \int_{\phi'}^{\infty} e^{-\theta^2} d\theta$ in
Logarithmic Form.

ϕ or ϕ'	0.001	0.002	0.005	0.01	0.02	0.05	0.1	0.2	0.3
$\log_{10} \phi$ or $\log_{10} \phi'$	3.0	3.301	3.699	2.0	2.301	2.699	1.0	1.301	1.477
$\log_{10} u$	6	6.602	5.398	4.0	4.602	3.399	2.0034	2.615	2.981
$\log_{10} u'$	4.947	3.2475	3.644	3.943	2.2385	2.6224	2.901	1.1559	1.291

ϕ or ϕ'	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.2	
$\log_{10} \phi$ or $\log_{10} \phi'$	1.602	1.699	1.778	1.845	1.903	1.954	0	0.0792	
$\log_{10} u$	1.250	1.471	1.663	1.836	1.999	0.155	0.307	0.610	
$\log_{10} u'$	1.377	1.436	1.480	1.513	1.540	1.561	1.580	1.606	

In order to find ζ by solving equation (62) above, we notice that

$$\phi' / \phi = \zeta / 2\sqrt{k'} \div \zeta / 2\sqrt{k} = \sqrt{k/k'}.$$

Hence

$$[\log \phi' - \log \phi] = \frac{1}{2} [\log k - \log k'].$$

k and k' being known, $[\log \phi' - \log \phi]$ can be calculated. Assuming the values we have employed hitherto, viz., $k = 4.5 \times 10^{-4}$, $k' = 6 \times 10^{-5}$, we find $[\log \phi' - \log \phi] = 0.438$. The simplest way to proceed is now to construct on a single sheet of paper two graphs in which

Y is $\log u$ and X is $\log \phi$

Y' is $\log u'$ and X' is $[\log \phi' - 0.438]$.

Knowing y_0 and λ we evaluate $[\log \lambda - \log y_0]$, which from equation (62) gives us $[\log u' - \log u]$, i.e., $(Y' - Y)$. We find on our graphs the points for which $(Y' - Y)$ has the required value for the same value of X. This gives us $\log \phi$, which is $\log \zeta / 2\sqrt{k}$. Hence ζ can be found and the boundary between the "oxygen" and the "lactic acid" zone moves forward according to the equation $\xi = \zeta\sqrt{t}$.

The rate at which oxygen is being used in oxidation is, at any moment,

$k(-dy/dx)_{x=\xi}$, which from equation (59) is $\frac{A\sqrt{k}}{2\sqrt{t}} e^{-\xi^2/4t}$. Substituting for A from equation (60) this becomes

$$\text{Rate} = \frac{y_0\sqrt{k}}{2\sqrt{t}} \frac{1}{e^{\xi^2/4k} \int_0^{\xi/2\sqrt{k}} e^{-\theta^2} d\theta},$$

which on introducing u and simplifying becomes

$$\text{Rate} = y_0 \zeta / 4u \sqrt{t}. \quad (63)$$

The total amount of oxygen used in time t is found by integrating (63), and is $y_0 \zeta \sqrt{t}/2u$, which may be written either $V = \xi y_0/2u$, or, employing equation (62) in the form $\lambda/y_0 = u'/u$, $V = \xi \lambda/2u'$. Thus,

$$V = \xi y_0/2u = \xi \lambda/2u'. \quad (64)$$

Thus the amount V of oxygen used in recovery to any depth ξ is

$$V = \frac{\text{oxygen debt in that depth.}}{2u'}. \quad (64A)$$

It can be shown that when $k' = 0$ the value of $2u'$ is 1, in which case we find, when lactic acid cannot diffuse, the obvious relation that the oxygen used in recovery to any depth is equal to the original oxygen debt of that depth.

Numerical Examples.—(1) Consider a plane sheet of muscle containing 0.1 per cent. lactic acid, so that $\lambda = 0.155$. Place this at 20°C . in oxygen, so that $y_0 = 0.031$. We wish to know the depth ξ of the recovery front, and the amount V of oxygen used per sq. cm. of surface, in time t . Here $\log \lambda - \log y_0 = 0.699$, so that we have to find the position on the curves of fig. 6 at which $\log u' - \log u = 0.699$. This is where $\log \phi = 1.40$. Here $\log u' = 1.52$. Hence $\phi = 0.251 = \zeta/2\sqrt{k}$; therefore $\zeta = 1.07 \times 10^{-2}$. Thus we may calculate,

t	= 1 min.	9 mins.	25 mins.	100 mins.
ξ	= 0.11 mm.	0.32 mm.	0.53 mm.	1.07 mm.

From equation (64) the amount V of oxygen used = $\xi \lambda/2u'$, $\lambda = 0.155$ and $u' = 0.33$; hence for the above times the amounts of oxygen are, respectively—

$$V = 0.0025 \text{ c.c.} \quad 0.0075 \text{ c.c.} \quad 0.0125 \text{ c.c.} \quad 0.025 \text{ c.c.}$$

Taking account therefore of the diffusion of lactic acid, the amount of oxygen used in recovery to any depth

$$= \frac{(\text{oxygen debt in that depth})}{2u'} = 1.5 \times (\text{original oxygen debt in that depth}).$$

(2) Similarly for 10 per cent. oxygen, but everything else the same, we have $\log \lambda - \log y_0 = 1.699$, $\log \phi = 2.62$, $\log u' = 2.95$. Hence $\phi = 0.042$, $u' = 0.089$. Thus $\zeta = 0.0018$, $\xi = 0.0018 \sqrt{t}$ cm. $= 0.018 \sqrt{t}$ mm. Thus in 100 minutes the depth is only 0.18 mm., and the volume of oxygen used in recovery to any depth is,

$$5.6 \times (\text{original oxygen debt in that depth}).$$

We see the greater importance of lactic acid diffusion at the lower oxygen pressure.

(3) Similarly for 1 per cent. oxygen. $\log \lambda - \log y_0 = 2.699$, $\log \phi = 3.68$, $\log u' = 2.06$. Hence $\phi = 0.0048$, $u' = 0.0115$. Thus $\zeta = 0.000204$, $\xi = 0.000204 \sqrt{t}$ cm. $= 0.00204 \sqrt{t}$ mm. Thus in 100 minutes the depth is only 0.0204 mm. The volume of oxygen used in recovery to any depth is $43 \times$ (original oxygen debt in that depth). We see here the extreme importance of lactic acid diffusion at very low oxygen pressures.

(4) For 100 per cent. oxygen, but a small oxygen debt, $\lambda = 0.0155$ (0.01 per cent. lactic acid) we have $\log \lambda - \log y_0 = -0.301$, $\log \phi = 1.88$, $\log u' = 1.66$. Hence $\phi = 0.76$, $u' = 0.46$. Thus $\zeta = 0.016$, and $\xi = 0.16 \sqrt{t}$ mm. Thus in 100 minutes the depth is 1.6 mm. The volume of oxygen used in recovery to any depth is $1.09 \times$ (original oxygen debt in that depth), showing that with a high oxygen pressure and a low oxygen debt the diffusion of lactic acid is of relatively small importance.

Approximate Solution for Low Oxygen Pressures.—In this case ζ remains small and the recovered zone increases only very slowly inwards. Hence we may write

$$e^{\zeta^2/4k} = e^{\zeta^2/4k'} = 1,$$

$$\int_0^{\zeta/2\sqrt{k}} e^{-\theta^2} d\theta = \zeta/2\sqrt{k}.$$

$$\int_{\zeta/2\sqrt{k'}}^{\infty} e^{-\theta^2} d\theta = \sqrt{\pi}/2.$$

Then we have, from equation (61),

$$\frac{\lambda}{y_0} = \frac{\frac{1}{\sqrt{k'}} \frac{\sqrt{\pi}}{2}}{\frac{1}{\sqrt{k}} \frac{\zeta}{2\sqrt{k}}} = \frac{k\sqrt{\pi}}{\zeta\sqrt{k'}}.$$

from which

$$\xi = \frac{ky_0}{\lambda} \sqrt{\frac{\pi t}{k'}}. \quad (65)$$

The total amount of oxygen used in time t is from equation (64) $V = \xi \lambda / 2u'$.

Substituting $u' = \frac{\xi}{2\sqrt{k'}} \times 1 \times \frac{\sqrt{\pi}}{2}$, this gives

$$V = 2\lambda \sqrt{\frac{k't}{\pi}} \quad (66)$$

which we see is independent of the diffusion constant of oxygen or of its concentration. It depends on the oxygen debt and on the diffusion constant of lactic acid. This, on reflection, is obvious. The oxygen concentration is low and consequently its gradient adjusts itself to the supply of lactic acid diffusing out. The low oxygen pressure is compensated by a steep gradient of concentration. The lactic acid comes as from a semi-infinite solid, where the amount diffusing out should be $2y'_0 \sqrt{k't/\pi}$ (see equation (49)), or in terms of its equivalent oxygen $2\lambda \sqrt{k't/\pi}$.

Thus in a muscle subjected to a low oxygen pressure there should be a recovery rate depending only on the oxygen debt and the time. *This assumes that a very thin layer of muscle is able to do all the oxidation necessary to recover from the onward diffusing lactic acid.* There must clearly be a limit to this ability; we cannot press the argument of this section very far, without knowing what is the limiting rate at which a muscle can carry on oxidative recovery. The following examples, however, are given for what they are worth, it being understood that in them we are making an unverified assumption, viz., that muscle can allow lactic acid and oxygen to react with one another as fast as these are brought together by diffusion.

Examples. --(1) If we take k' as 6×10^{-3} , then $2\sqrt{k'/\pi} = 8.7 \times 10^{-3}$, and the amount of oxygen used in time t is, from equation (66), $0.0087\lambda\sqrt{t}$ c.c. per sq. cm. of surface. If we take λ' as the oxygen debt *per mm. depth per sq. cm. surface* and t as 9 minutes, the amount of oxygen becomes $0.26\lambda'$. Thus, considering a muscle 1 mm. thick, one quarter of the recovery has gone on in 9 minutes, because of the lactic acid diffusion, and in spite of a low oxygen pressure.

(2) The depth of the recovered zone is given by (65) above. Taking the case of 0.005 per cent. of lactic acid (approximately the amount liberated in a 1-second tetanus) for which $\lambda = 0.0078$, and 1 per cent. oxygen ($y_0 = 0.00031$), we find $\xi = 4.1 \times 10^{-3}\sqrt{t}$. Thus in 1 minute $\xi = 41 \mu$, in 25 minutes $\xi = 205 \mu$. The thickness of an ordinary frog's sartorius muscle being about 600μ , this shows that 1 per cent. oxygen can allow an appreciable degree of recovery to go on. The amount of oxygen used in time t is $2\lambda\sqrt{k't/\pi}$. For

$t = 25$ minutes this is 3.38×10^{-4} . The oxygen debt of 600μ is $0.06 \times 0.0078 = 4.7 \times 10^{-4}$ c.c. Thus in 25 minutes in 1 per cent. oxygen, the recovery of a muscle 0.6 mm. thick after a 1 second tetanus is about two-thirds complete. This is due largely to the diffusion of the lactic acid.

(b) *Sheet of Finite Thickness.*—If the lactic acid be regarded as incapable of diffusing (Case I) the course of the recovery process will be the same whether the sheet be of finite or infinite thickness. The "oxygen" zone will simply increase in depth as the square root of the time, until the whole sheet has recovered. If, however, the lactic acid can diffuse, the progress of recovery may be substantially different in the later stages if the sheet be of finite thickness. There appears to be no reasonably simple solution of the general problem with a sheet of finite thickness. It seems likely, however, that for the greater part of the process the same constant value of ξ/\sqrt{t} holds, as in the cases discussed already. When oxygen diffusion alone counts (Case I) ξ/\sqrt{t} is constant up to the end. When lactic acid diffusion alone counts, the problem can be solved (as in Part II) by the aid of a Fourier series, and it is found that ξ/\sqrt{t} is constant within 2 per cent. until two-thirds of the recovery is complete. Presumably the general case lies somewhere between these two, and it seems likely that the formula $\xi/\sqrt{t} = \text{a constant}$ is accurate enough over the major portion of the process. Thus we can determine ξ from equation (62), and assume that $\xi/\sqrt{t} - \xi$ is true up to about two-thirds of complete recovery.

§ 2. DIFFUSION OF OXYGEN INTO A CYLINDER, IN WHICH RECOVERY IS NECESSARY BEFORE THE OXYGEN CAN ADVANCE.

CASE I.—OXYGEN ALONE DIFFUSING: ACCOUNT TAKEN OF METABOLISM.

This case has not proved soluble in general on the lines of (A) Case I, and we will treat it in the same way as we followed for the approximate equations (55) to (57) above, viz., by assuming that the oxygen front moves forward so slowly that the oxygen concentration behind it has time to attain a steady state. This will be a good approximation when the oxygen debt is large compared with the oxygen concentration. In the oxygen region then we have (see equations (22) and (23) above)

$$a = k \frac{d}{dr} \left(r \frac{dy}{dr} \right),$$

from which

$$y = ar^2/4k + B \log r + E.$$

Now at $r = r_0$ $y = y_0$, and at $r = \rho$ (the radius of the oxygen front at any time) $y = 0$. Hence

$$y_0 = ar_0^2/4k + B \log r_0 + E$$

$$0 = a\rho^2/4k + B \log \rho + E,$$

so that

$$B = \frac{y_0 - a(r_0^2 - \rho^2)/4k}{\log r_0/\rho}.$$

Now if λ_0 be the initial oxygen debt, $\lambda = (\lambda_0 + at)$ is the oxygen debt at time t in the inner region, and the rate at which the oxygen debt is being paid off is $(\lambda_0 + at)(-d\rho/dt)$. This must be equal to the rate of diffusion of oxygen up to the boundary, $k(dy/dr)_{r=\rho}$; carrying out the differentiation, putting $r = \rho$, and substituting for B we find,

$$(\lambda_0 + at) \frac{d\rho}{dt} = \frac{\frac{a}{4}(r_0^2 - \rho^2) - y_0k - \frac{a\rho^2}{2} \log r_0/\rho}{\rho \log r_0/\rho}.$$

This equation can be integrated in the form.

$$1 + \frac{at}{\lambda_0} = \frac{1}{1 - \frac{ar_0^2}{4y_0k} \left[1 - \frac{\rho^2}{r_0^2} \left(1 + \log \frac{r_0^2}{\rho^2} \right) \right]}.$$

Putting $\rho^2/r_0^2 = 1 - \theta$, as in Part I, so that θ is the fraction of the cylinder in which recovery has proceeded, and rearranging, this becomes,

$$\theta - (1 - \theta) \log_e \left(\frac{1}{1 - \theta} \right) = \frac{4y_0kt/r_0^2\lambda_0}{1 + at/\lambda_0}. \quad (67)$$

When the metabolism of the tissue can be neglected this becomes

$$\theta - (1 - \theta) \log \left(\frac{1}{1 - \theta} \right) = 4y_0kt/r_0^2\lambda_0. \quad (68)$$

The values of the function $u = \theta - (1 - \theta) \log \left(\frac{1}{1 - \theta} \right)$ were given in Table I above. These can be used for the numerical solution of equations (67) and (68). It should be noted that t is infinite when

$$\theta - (1 - \theta) \log \left(\frac{1}{1 - \theta} \right) = 4y_0k/ar_0^2.$$

This is the same as equation (31) and gives the degree of penetration of oxygen during the steady state.

Note.—The time t for any given degree of recovery is directly proportional to the initial oxygen debt λ_0 .

Numerical Examples.—For a resting muscle at 20° C. in oxygen $k = 4.5 \times 10^{-4}$, $a = 7 \times 10^{-4}$, $y = 0.031$; for 0.1 per cent. lactic acid $\lambda_0 = 0.155$. The following table gives times for various degrees of recovery for cylindrical muscles of different diameters, calculated (i) from equation (67) (resting metabolism taken into account) and (ii) from equation (68) (metabolism neglected).

Extent of recovery.	Taking account of metabolism.			Taking no account of metabolism.		
	10 per cent.	50 per cent.	100 per cent.	10 per cent.	50 per cent.	100 per cent.
Time, minutes						
$r_0 = 1$ mm.	0.147	4.3	31.5	0.147	4.3	27.8
$r_0 = 1.5$ mm.	0.33	9.7	87	0.33	9.7	62.7
$r_0 = 2.5$ mm.	0.93	30.5	807	0.93	27	174
$r_0 = 3.16$ mm.	1.47	53.4	No solution	1.47	43	278

We see that for the smaller extents of recovery and for the smaller radii the resting metabolism of the tissue has little effect on the rate at which the oxygen front advances; under other conditions, however, it may exert a preponderating effect.

CASE II.—LACTIC ACID DIFFUSING AS WELL AS OXYGEN.

This case has not proved soluble in general: we can only proceed by approximation and analogy. In the plane case we found an approximate formula (equation (55)), $\xi^2/t = 2ky_0/\lambda$, for the depth of recovery at time t , when the diffusion of oxygen alone was considered. Taking account of the diffusion of lactic acid required $2ky_0/\lambda$ in this formula to be replaced by ζ^2 , where ζ is the solution of equation (62). Now for a cylinder we found, in the case of oxygen alone diffusing, the approximate formula (equation (68))

$$\theta = (1 - \theta) \log \left(\frac{1}{1 - \theta} \right) = 4ky_0t/\lambda r_0^2.$$

For short times the cylindrical solution must be the same as the plane, and equations (55) and (68) agree for short times. Equally the equations for lactic acid diffusing must be the same for short times. Now in the plane case the diffusion of lactic acid was taken account of by replacing $2ky_0/\lambda$ by ζ^2 .

Presumably therefore we shall obtain a good approximation for the cylindrical case by the same means, so that equation (68) becomes

$$\theta - (1 - \theta) \log \left(\frac{1}{1 - \theta} \right) = 2\gamma^2 t / r_0^2. \quad (69)$$

This equation is presumably a fair approximation up to about two-thirds of complete recovery.

CASE III. - THE DIFFUSION OF OXYGEN OUTWARDS FROM A BLOOD VESSEL INTO A REGION SHOWING AN OXYGEN DEBT.

The case of cylindrical diffusion outwards *during a steady state* has been discussed already (equations (29) and (30)). Krogh (1) employed equation (29) in studying the supply of oxygen to a tissue intersected with a number of blood capillaries, supposed—for simplicity—to be uniformly distributed throughout it. The average partial pressure of oxygen in the blood of the capillary ($r = r_0$) being y_0 , and diffusion being supposed to end at $r = r_1$, the least partial pressure in the tissue, that at $r = r_1$, is given by

$$\frac{y_1}{y_0} = 1 - \frac{ar_0^2}{4ky_0} \left[\frac{r_1^2}{r_0^2} \log \frac{r_1^2}{r_0^2} - \frac{r_1^2}{r_0^2} + 1 \right].$$

Putting $\theta = (r_1^2/r_0^2 - 1)$ this becomes

$$\frac{y_1}{y_0} = 1 - \frac{ar_0^2}{4ky_0} [(\theta + 1) \log_e (\theta + 1) - \theta]. \quad (70)$$

where θ represents the area of the tissue divided by the area of the blood vessel.

Table VII.—Values of $u = (\theta + 1) \log_e (\theta + 1) - \theta$.

θ	0	0.2	0.5	1	1.5	2	3	5	7	10
u	0	0.0188	0.109	0.386	0.791	1.297	2.544	5.752	9.632	16.380

θ	15	20	25	30	40	50	70	100	150	200
u	29.35	44.00	59.7	76.3	112.2	150.3	232.5	366.5	608	866

The data of Table VII are shown graphically in fig. 7.

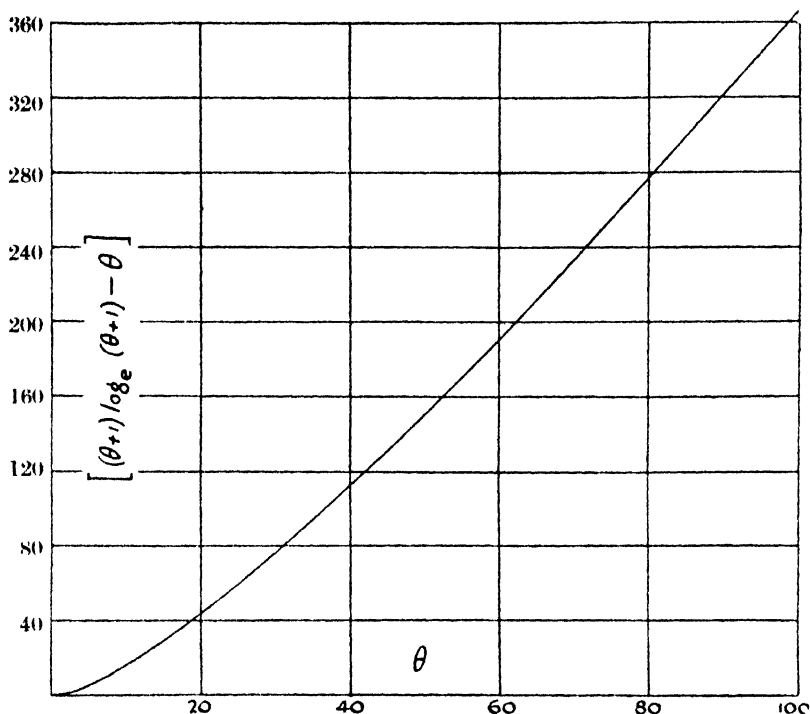


FIG. 7.- Graph of $[(\theta + 1) \log_e (\theta + 1) - \theta]$ as a function of θ . θ represents the area supplied with oxygen by a blood capillary, divided by the area of the capillary.

The maximum rate of oxygen consumption during a steady state of severe exercise in an athletic man of 75 kilograms weight is about 4.5 litres per minute. If this oxygen be used, as in running, by (say) $22\frac{1}{2}$ kilograms of muscle, each cubic centimetre of muscle must use about 0.2 c.c. of oxygen per minute, $a = 0.2$. According to Krogh (1) the diameter of the actual capillaries is of the same order of size as that of the red corpuscles; if we take 8μ as the diameter ($r_0 = 0.0004$), $k = 1.64 \times 10^{-5}$ (Krogh's value for 37°C.), and y_0 as the oxygen partial pressure of the venous blood, say 0.01 atmosphere, then $4ky_0/ar_0^2 = 20.5$. Now if an oxygen debt is not to be set up anywhere in the tissue, the whole of the latter must have a partial pressure of oxygen greater than zero. Thus y_1 must be > 0 . The limiting case when $y_1 = 0$ is given (from equation (70)) by $(\theta + 1) \log (\theta + 1) - \theta = 20.5$: from fig. 7, $\theta = 12$ approximately. Thus each blood capillary must supply oxygen to about 12 times its own volume of muscle. If $22\frac{1}{2}$ kilograms of muscle be involved, then at this extreme rate of working there must be about 1.9 litres of blood in its capillaries. The total area of blood plus tissue for

each capillary is, according to this calculation, 13 times the area of the capillary, viz., 655 sq. μ . Thus there should be about 1500 capillaries open in every sq. mm. of tissue. Krogh by microscopic examination of the semi-membranous of the dog found a total of about 2600.

If the work undertaken be more severe than can be accounted for by the maximum oxygen supply, an oxygen debt is set up, owing to the accumulation of lactic acid. Imagine this oxygen debt to be suddenly produced by exercise of extreme violence, *e.g.*, by a 200 yards "sprint." It is interesting to calculate the time in which the debt can be paid off, assuming that the time depends only on the diffusion of the necessary oxygen.

As before y_0 is the constant oxygen concentration at the circumference of the blood vessel, and the oxygen debt λ in the unrecovered region is assumed to increase uniformly with the time, $\lambda = \lambda_0 + at$. In the recovered region there is a "basal" oxygen consumption a . The whole treatment is similar to that by which equation (67) above was derived, except that ρ is now greater than r_0 . The solution is therefore

$$u = (\theta + 1) \log_e (\theta + 1) - \theta = \frac{4y_0kt/r_0^2\lambda_0}{1 + at/\lambda_0} \quad (71)$$

or

$$t = \frac{\lambda_0 r_0^2 u}{4ky_0 - ar_0^2 u} \quad (72)$$

where $\theta = (\rho^2/r_0^2 - 1)$, and represents, as a fraction of the area of the capillary, the tissue area which has recovered in time t . Table VII and fig. 7 above give u as a function of θ . Knowing θ , y_0 , k , λ_0 , a , r_0 , it is possible to calculate the time required for complete recovery.

An oxygen debt of 10 litres in a 75 kilogram man, assumed to occur in 25 kilograms of muscle, is about 0.4 c.c. per c.c. of muscle, $\lambda_0 = 0.40$. The resting metabolism of the whole man being about 250 c.c. per minute, the metabolism of the 25 kilograms of muscle in question must be about 125 c.c., *i.e.*, $a = 0.005$. We will take r_0 as $4\mu = 4 \times 10^{-4}$ cm. Complete recovery may be to 40 times the area of the blood vessel (it is assumed that during recovery not so many capillaries will be open as during exercise with its much larger blood flow) so that $\theta = 40$. This gives $(\theta + 1) \log (\theta + 1) - \theta = 112$. Assuming k for 37° C. to be 1.64×10^{-5} in Krogh's units, and y_0 to be 0.015 atmosphere, and employing equation (72), we find

$$t = \frac{7.17 \times 10^{-6}}{9.8 \times 10^{-7} - 9 \times 10^{-8}} = 8 \text{ minutes approximately.}$$

In actual fact observation of the oxygen consumption after a sharp burst of severe exercise has shown that recovery is 80 per cent. or more complete within 8 minutes. The remainder may be due to lactic acid which escaped early from the active muscles into the general circulation, as suggested by Hill, Long and Lupton (12). It may, however, be due to the gradual closing down of the capillaries as recovery proceeds.

The time required for recovery, other things being the same, increases proportionally to the oxygen debt λ_0 . It increases also with the area served by each capillary (since u increases with θ), so that the later stages of recovery in a subject remaining at rest after exercise may be very protracted, owing to the closing down of a large fraction of the blood capillaries in the muscle. It is proportional to y_0 , the average partial pressure of oxygen in the capillary; this emphasises the advantage of a rapid blood flow during recovery, which can be aided by gentle exercise. It depends to some degree on the metabolism of the tissue, to a greater degree if u or r_0 be large: when the majority of the capillaries are closed and u therefore is great the metabolism factor $\sigma r_0^2 u$ in the denominator of equation (71) may become very important.

The striking dependence of the time of recovery in equation (71) on the radius of the capillary, varying almost as its square (assuming that the value of θ remains constant), emphasises the most important factor in the design of the circulation, namely, the existence of a very large number of capillary vessels of very small size. For a given volume of capillary blood in the tissues, the time required for diffusion and recovery varies as *the square* of the average diameter of the vessels. Krogh's equation for a steady state, and its extension to the dynamic state in equations (70) and (71), together with a knowledge of the numerical values involved, give us a quantitative picture of the necessities of the capillary circulation which is unattainable except by such means.

Mr. A. D. Ritchie has pointed out to me that an argument similar to that given here, in relation to the size of blood capillaries, may be applied to the case of muscle fibres: that is to say, the maximum diameter of a muscle fibre is probably not fixed by mechanical factors but by the speed of diffusion.

PART IV.—THE DIFFUSION OF CARBON DIOXIDE THROUGH A TISSUE.

Krogh (5) gives a value for the coefficient of diffusion of carbon dioxide through tissue, which depends, however, only on a single observation. Expressed in Krogh's units the coefficient for carbon dioxide is about 36 times that for oxygen; this, of course, depends on its great solubility; expressed in ordinary

units, *i.e.*, after dividing by the ratio of the solubilities, it would be only 1.2 times that for oxygen. It seems curious that the larger molecule should diffuse faster; we might have expected it to diffuse only 0.8 times as fast. Assuming that Krogh's single value is correct, the explanation is probably as follows. Carbon dioxide in solution in a tissue is largely in the form of bicarbonate, every tissue having a characteristic CO_2 -dissociation curve analogous to that of blood. Thus, if the bicarbonate ion is able to diffuse, the diffusion of total carbon dioxide may be more rapid than that of free carbon dioxide at the same gradient of partial pressure.

It is not possible, with the limited information at present available, to discuss quantitatively the diffusion of carbon dioxide through a tissue of given shape and size. Certain generalisations, however, may be made. Let Y be the concentration of combined carbon dioxide when y is that of free carbon dioxide. Then Y is related to y by an equation, (say) $Y = f(y)$. Let us assume first that the bicarbonate ion is incapable of diffusing through living tissue. Then, for plane diffusion, since y and Y increase or decrease together (by chemical combination or breakdown), while free carbon dioxide alone diffuses, the following equation must hold,

$$dy/dt + dY/dt = k d^2y/dx^2$$

or

$$(1 + df(y)/dy) dy/dt = k d^2y/dx^2.$$

This equation is not in general soluble. Over a narrow range, however, of carbon dioxide pressures, especially in the zone of physiological importance (30 to 50 mm. Hg.), Y may be taken approximately as a linear function of y , say $Y = p + qy$. Our differential equation then becomes

$$(1 + q) dy/dt = k d^2y/dx^2.$$

Let z = the concentration of total carbon dioxide

$$= Y + y = p + (q + 1) y.$$

Then $dz/dt = (q + 1) dy/dt$ and $d^2z/dx^2 = (q + 1) d^2y/dx^2$.

Hence we may write

$$dz/dt = [k/(1 + q)] d^2z/dx^2.$$

Thus the diffusion constant of total carbon dioxide is $k/(1 + q)$, which may be considerably less than k . For a given *concentration gradient*, therefore, the diffusion constant of total carbon dioxide is less than that of free carbon dioxide, in a tissue which does not permit the diffusion of the bicarbonate ion. On the other hand, for a given *partial pressure gradient* (the total carbon dioxide dissolved being greater than the free carbon dioxide) the diffusion constant is

the same for total as for free carbon dioxide. If Krogh's single observation be correct, the fact that the diffusion constant is greater than we should expect shows that the bicarbonate ion is, in fact, capable of diffusing.

Suppose now that the bicarbonate ion has a diffusion constant K . Then, as before

$$dy/dt + dY/dt = k d^2y/dx^2 + K d^2Y/dx^2.$$

Assuming again, as a rough approximation, that $Y = p + qy$, this becomes

$$(1 + q) dy/dt = (k + qK) d^2y/dx^2,$$

or introducing z , as before

$$dz/dt = [(k + qK)/(1 + q)] d^2z/dx^2.$$

Thus, working in *concentrations* (not partial pressures), the absolute diffusion constant for total carbon dioxide is $(k + qK)/(1 + q)$, which is greater, or less, than k according as K is greater or less than k . It seems unlikely, on general grounds, that the charged ion should penetrate the membranes of the cell more rapidly than the uncharged molecule; certainly other anions, viz., lactate and phosphate, do not. In that case K is less than k and the absolute value of the diffusion constant of total carbon dioxide will be less than that of free carbon dioxide.

For a given *partial pressure gradient*, in this case, more total carbon dioxide will diffuse if the bicarbonate ion be capable of diffusing than if it be not, in the ratio of $(k + qK) : k$. This indeed is obvious on general grounds. The high value found by Krogh supports the idea that the bicarbonate ion can diffuse.

If we may assume that the limits of concentration of carbon dioxide in the tissue involved are such that the CO_2 dissociation curve is approximately a straight line within the range considered, then we may apply the same treatment as has been developed for other substances in the above pages to the total carbon dioxide. Before doing so, however, we require to know the diffusion constant for total carbon dioxide *through the tissue in question, within this range*. Since no information on this essential point is available at present, the subject cannot, with profit, be further studied here.

My sincere thanks are due to Mr. P. E. Marrack, of the Admiralty, for his assistance in the mathematical part of this work. The solutions of the problems discussed in Part III, §§ 1 and 2 are due to him, and various suggestions of his have been adopted elsewhere. To Prof. G. B. Jeffery, F.R.S., of University College, London, also I am indebted for help and advice.

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Address of the President, Sir Ernest Rutherford, O.M., at the Anniversary Meeting, November 30, 1928.

During the past year the losses to our Society by death have been unusually heavy, including three Foreign Members, thirteen Fellows, and two Fellows elected under Statute 12, the Earl of Oxford and Asquith and Viscount Haldane. While the main work of Lord Haldane lay in legal and administrative fields, he always had a deep interest in science and its philosophy, and gave much valuable help to the cause of University Education.

The death of Prof. P. H. VON GROTH, Foreign Member of our Society, at the age of 84, removes a notable figure from the Science of Mineralogy. Distinguished as investigator and writer and editor for 39 years of the 'Zeitschrift für Krystallographie und Mineralogie,' he widely influenced the development of his science, and by his own researches and through his students made notable contributions to our knowledge.

The death of HENDRIK ANTOON LORENTZ, Foreign Member of the Royal Society and Nobel Laureate, has deprived physical science of one of the greatest figures of our time. He will be remembered mainly by his extension of Maxwell's electromagnetic theory. He introduced a new precision into this theory, and brought it into line with current experimental physics, by breaking up Maxwell's electric charges into crowds of electrons. Especially noteworthy was his explanation of the newly-discovered Zeeman Effect, in terms of electronic motion inside the atom. Contemporaneously with Larmor, he examined and discussed the effects produced by the motion of an electric system through "the ether." This part of his work culminated in a proof that the effect of this motion could be exactly reproduced by a modification of the measures of time and space in the system. The equations which embody this result, universally known as "Lorentz's transformation," ultimately formed the foundation stone of the Theory of Relativity.

In his latter years, he naturally and by general consent took the leading place in every Continental congress of physicists. Those who have seen him

presiding over such functions can never forget his unfailing tact and courtesy, his geniality and kindness to all, his ready wit, his command both of language and languages, and the vast store of scientific knowledge which was ever held in reserve, to be brought into service when needed.

At the request of the Society, I attended the funeral of Lorentz at Haarlem, which was national in scope and testified to the high place he held in the esteem of all sections of his countrymen. Eulogiums on the work and personality of Lorentz were spoken at the graveside by Prof. Ehrenfest, Langevin, Einstein and myself.

By the death of W. H. DINES, at the age of 73, Meteorology has lost an outstanding pioneer in the study of the upper atmosphere. By means of kites and balloons and measuring apparatus of entirely his own design, he was one of the first to make a systematic study of the meteorological state of the upper air. For 20 years Director of Experiments in the Upper Air for the Meteorological Office, he rendered great services to the Science he loved.

SIR AUBREY STRAHAN, who died on March 4th, was till 1920 Director of the Geological Survey of Great Britain and a leading authority on the stratigraphical Geology of this country, and especially on that of the coalfields. He had been President of the Geological Society of London, and a member of the Royal Commission on Coal Supplies. Elected to our Fellowship in 1903, he gave valued service on the Council and Committees of the Society, acting in the last months of his life as Chairman of a Committee appointed to draft a report on Museums and Public Galleries.

In SIR DAVID FERRIER, who has died in his 87th year, we have lost a pioneer investigator of the physiology of the brain, whose work laid the experimental foundation of all modern knowledge of the localised functions of the cerebral cortex, and thereby opened a new epoch in the medical diagnosis of disease or injury of the brain, and in their relief by surgical operation. Ferrier was one of the founders of the Physiological and of the Neurological Society, and one of the small group who, in 1878, started the Journal 'Brain,' to deal with neurology in its widest experimental and clinical aspects. For many years an honoured and successful physician, he preserved to an advanced age an enthusiastic interest in the advance of neurology by the experimental method. In 1906-1908 he was a Vice-President of the Society, of which he had been a Fellow for 52 years at the time of his death.

Science has suffered a severe loss by the early death of THEODORE W. RICHARDS, Director of the Gibbs Memorial Laboratory, Harvard University,

Nobel Laureate in Chemistry, Foreign Member of our Society and Davy Medallist. He early devoted himself to the accurate determination of the atomic weights of the elements and introduced many new methods leading to much increased precision in the measurement of these fundamental constants. The value of his work has been universally recognised, and his laboratory at Harvard attracted many research students from all parts of the world. He was much interested in the compressibility of atoms and molecules and made many accurate measurements in this field. A man of wide interests and sympathies, he had many friends in this country.

HANS FRIEDRICH GADOW, of German birth, but of British nationality since 1884, was for many years lecturer and reader in the advanced morphology of vertebrates in the University of Cambridge. His researches and writings on vertebrate anatomy, and especially on that of birds, reptiles, and amphibia, have had a great and stimulating influence on zoological thought. A naturalist as well as an anatomist, he sought every opportunity of seeing the forms which he studied under natural conditions, keeping many alive in his garden and wandering as far afield as the caves and mountains of Northern Spain and of Mexico. A man of robust and enthusiastic temperament he is held in grateful memory by many Cambridge men as a popular and influential teacher.

JOHN HORNE, a former Assistant Director of the Geological Survey of Great Britain, was in charge of the Geological Survey of Scotland from 1901 until 1911. In association with different colleagues he was responsible for important memoirs on the structure of the North-West Scottish Highlands and on the Silurian rocks of Scotland. At the time of his death, at the age of 80 years, he was still engaged in the completion of a work on the Geology of Scotland, begun in collaboration with the late B. N. Peach.

Sir JOHN THORNYCROFT, who died in his 85th year, became a Fellow of the Society in 1893. A pupil at Glasgow of Kelvin and Rankine, he began the construction of small high-speed craft on the Thames almost simultaneously with Sir Alfred Yarrow. In 1877 he built for the Admiralty his first torpedo boat, followed in rapid succession by other craft, including, in 1907, the ocean-going destroyer "The Tartar," driven by Parsons turbines at 35.6 knots. In time his yard was moved to Southampton, and from it during the War was launched a stream of destroyers and flotilla leaders. During his later years he devoted himself to some of the problems which troubled the builder of high-speed vessels, utilising for the purpose a small tank constructed in his grounds.

CHARLES CHREE, after graduating in the University of Aberdeen, came to

Cambridge, and was 6th Wrangler in 1883, gaining a Fellowship in King's College in 1885. In 1893 he was appointed Superintendent of the Kew Observatory, and filled this post with much distinction until his retirement in 1925. He published many original papers on subjects connected with terrestrial magnetism and atmospheric electricity and elasticity. Of international reputation, the value of his work was recognised by the award to him of the Hughes Medal of the Society.

SIR HORACE DARWIN, son of Charles Darwin, was educated as an engineer. He will long be remembered as the man who, by the application of engineering principles, transformed the industry of scientific instrument making in this country. Soon after Michael Foster went to Cambridge as teacher in physiology, Darwin began, in partnership with Dew Smith, to make instruments for the new laboratory. The work grew, and in time developed into the Cambridge Scientific Instrument Company, with Darwin as its chairman and inspiring genius. Many of the instruments were of his own design, and practically all bore the impress of his supervision. He was one of the first to apply the principles of geometrical design laid down by Kelvin and Maxwell. The changes in instrument making during the past fifty years have been far reaching. Darwin was a leader in this advance, a friend ever ready to help those who came to him for advice.

DIARMID NOËL PATON, for 22 years Regius Professor of Physiology in the University of Glasgow, was an eminent and successful teacher and an enthusiastic investigator of problems drawn from a wide range of his subject. He was among the earliest of British physiologists to investigate metabolic problems and in more recent years had devoted, with his pupils, many years of experimental study to the function of the parathyroid glands. In an age of increasing specialisation he remained conspicuous for the width of his knowledge and interest in physiology and in its practical application to sociology and medicine.

G. H. BRYAN was an applied mathematician of distinction. His early work lay in the fields of hydrodynamics, thermodynamics and the kinetic theory of gases. Later he turned his attention to the problem of aviation; he was the first to investigate stability of flight by treating the moving aeroplane as a system undergoing small oscillations about a state of steady motion, thus laying the foundations of the theory of stability in aviation.

By the death of Sir CHARLES TOMES, in his 83rd year, the Society has lost from its Fellowship a great pioneer in dental anatomy. In his continuation of the work of his father, also one of our Fellows, he may be said to have founded the science of comparative dental histology. As a member and Treasurer

of the General Medical Council, he was largely responsible for raising the standard of scientific training for the practice of dental surgery.

Sir ALEXANDER KENNEDY, who has recently passed from us, had been for over forty years a Fellow of our Society. In 1874 he became Professor of Engineering in University College, Gower Street, and established there the first Engineering Laboratory in this country, devising a method of education which has been followed widely both at home and abroad. He was one of the first to realise the need for placing structural engineering on a sound mechanical basis and was largely responsible for the introduction of testing machines. On resigning his professorship in 1889, he commenced practice as a consulting engineer and in that capacity designed electrical works for power and lighting in many parts of the Empire. He was a man of many interests and, of late years, by his photographic records of the ruins of Petra made known to the world some of the wonders of the civilisation of which it was the centre.

Sir HUGH KERR ANDERSON, Master of Caius College, Cambridge, since 1912, and a Fellow of our Society since 1907, was a member of our Council at the time of his recent death, at the early age of 63. Returning to Cambridge in 1891, on the completion of his medical course in London, he devoted himself during the next 14 years, with self-sacrificing zeal and conspicuous success, to research and teaching in his chosen subject of physiology. He was one of the investigators who were then unravelling the intricacies of the autonomic system and shaping the conceptions of its function now everywhere accepted. The series of classical papers in which he was associated with the late Prof. Langley, and his later and independent work on the complex reactions of the pupil of the eye, had shown him to the world of science as a coming master in his subject; while to every Cambridge man who in that period entered upon research or higher study in physiology, Anderson had been lavish in his gift of interest, encouragement and personal help. From 1905 onwards the recognition of his aptitude for business drew him, always diffident and reluctant, away from research into the wider affairs of his College and of his University. As a member of the Royal and Statutory Commissions, and in many other capacities, he did inestimable service to Cambridge, to science, and to education, and earned the honour and affection of a wide circle beyond his University and our Society.

Turning to other matters, I shall say a few words on events of interest to our Society during the year. After the normal period of office, Sir Richard Glazebrook retires to-day from the position of Foreign Secretary, and I

am glad to take this opportunity to convey to him the gratitude of the Society for his valuable services. In a difficult time, he has very efficiently represented our Society at the meetings of the International Research Council and has been indefatigable in his correspondence on international questions. We are fortunate to secure as his successor Sir Henry Lyons, who, in association with Sir Richard Glazebrook, has represented the Society at International meetings. His wide experience in this direction had led to his appointment as Secretary of the International Research Council in succession to Sir Arthur Schuster, who has recently retired from this important office. Since its foundation, he has been General Secretary of the Union for Geophysics.

Before the meeting on June 21, I had the pleasing task of accepting on behalf of the Society a portrait by Mr. R. G. Eves of our former President, Sir Charles Sherrington, which was presented by Sir John Rose Bradford on behalf of the subscribers. The Society is proud to possess this portrait, which is now hanging on our walls, and I think all will agree that it is an excellent likeness of my distinguished predecessor. It will serve as a permanent memento of one whose great and still continued services to Science and our Society we all gratefully recognise.

I have already referred to the loss of that veteran investigator Sir DAVID FERRIER. His friends and admirers have collected a fund of £1,000 which they have presented to the Society for the foundation of a Lecture in his memory. The Society has gratefully accepted this offer, so that in future on the biological side in addition to the well-known Croonian Lecture, there will also be a "David Ferrier Lecture" to be given at intervals and on subjects to be determined later.

It will be remembered that in the year 1922 our Society made a new departure by instituting Royal Society Professorships. With the aid of the Foulerton Fund, bequeathed to the Society, and the munificent gift of Sir Alfred Yarrow, we have been able in the last few years to appoint five professors, two Foulerton Professors in the medical sciences and three Yarrow Professors in the physical sciences. The main object of our Society is the "Improvement of Natural Knowledge" by discovery, and the main line of discovery must depend upon research. The Society has endeavoured to attain this object in two ways, first through its Studentships or Fellowships, six in number, including the Sorby, Foulerton, Mackinnon, Moseley, Tyndall and Armourers' and Brasiers', awarded to younger men who have shown marked promise as investigators, and secondly, through its Professorships, which are awarded to those who have shown over a longer period an unusual original capacity for advancing know-

ledge by experiment. The holders of these Professorships are expected to devote their whole attention to research and are freed from all duties of routine teaching and administration.

As the Royal Society has no laboratory of its own these Professorships may be held in any scientific institution which is in a position to offer suitable research facilities. A satisfactory feature of this scheme of administration lies in the fact that the Royal Society does not compete with existing institutions, but co-operates with and reinforces them. In all cases so far arrangements have been made for our Professors to continue their work in the same laboratories as before, and we are grateful to the Universities and Institutions concerned for the cordial and generous way in which they have co-operated with the Royal Society for this purpose.

The creation of Research Professorships by the Society was in the nature of an experiment, but I think all will agree that the experiment has proved an unqualified success. We have been fortunate in being able to attract some of our ablest and most original workers, who have devoted themselves to their investigations with zeal and enthusiasm, as evidenced by the high quality of their accomplished work. As it is now six years since the institution of the first professorship it has been thought desirable that I should give a brief review of some of the work carried out by our Professors. This is a difficult task in a limited time, but I hope I shall be forgiven for any shortcomings.

Prof. A. Fowler, who was appointed Yarrow Professor on December 1, 1923, has continued his researches in the Astrophysics Laboratory of the Imperial College of Science and Technology. Everywhere recognised as one of the most skilful of our experimenters in spectroscopy, he has during his tenure of office contributed to our 'Proceedings' a number of important papers, which have widely extended our knowledge of the modes of vibration of atoms under different conditions of excitation, when they have been deprived of one or more of their normal number of electrons.

His attention has been directed mainly to the production and analysis of the spectra of some of the lighter elements at successive stages of ionisation, including carbon, nitrogen, oxygen and silicon. These spectra are not only of importance from the theoretical standpoint, but also in their application to the interpretation of the spectra of the hotter stars. He has obtained data of precision over a wide range of experimental conditions, and covering a range of spectrum from the extreme ultra-violet to the infra-red. The investigation of these spectra has presented considerable experimental difficulty, especially in connection with the elimination of impurities. The results obtained for

nitrogen and oxygen have already found an application of special interest, in the use which has been made of them by Bowen in his important interpretation of previously unidentified lines in the spectra of gaseous nebulae.

During the tenure of his professorship Prof. Fowler has also been charged with the direction of the work of a number of research students and assistants at the Imperial College, some of whom have published valuable papers on spectroscopic subjects.

Prof. G. I. Taylor, who was appointed Yarrow Professor on December 1, 1923, continued his investigations in the Cavendish Laboratory, Cambridge. Prof. Taylor possesses that unusual combination of power of detailed mathematical analysis coupled with an ability to design and carry out difficult experiments. He has been specially interested in studying the modes of deformation of single crystals when subjected to uniform strain. The ordinary engineering tests applied to a specimen of metal merely give the average stress over a large number of crystal grains. The discovery of methods of producing single crystals of metals has made it possible for the first time to know the direction and magnitude of the stress throughout the crystal. Prof. Taylor and his co-workers have now determined the relationship between stress, strain and crystal axes for aluminium, iron and some other metals, with most interesting and important results. This work has been extended by other investigators at home and abroad, and to include cases where the stress is alternating.

I can only refer in passing to his continued interest in problems of hydrodynamics, where he has made calculations leading to predictions concerning fluid motion and the behaviour of bodies immersed in fluids. These have been verified by simple experiments shown before our Society. Recently he has turned his attention to the effect of compressibility on the flow of air past bodies moving at high speeds. This problem is now acquiring great importance in aeronautics owing to the rapidly increasing speed of aircraft, but difficulties of mathematical analysis have so far prevented more than very limited advances towards a dynamical theory. Prof. Taylor has now invented a machine, a kind of mechanical mathematician, by means of which problems of flow can be solved, which have so far defeated ordinary human mathematicians. He is now applying this machine to problems of practical importance in aeronautics.

Prof. O. W. Richardson, who was appointed third Yarrow Professor on August 1, 1924, continued to work in the laboratory of King's College, London. Like Prof. G. I. Taylor, he is equally at home in the theoretical and experimental side of physics. His main work in the last few years has been connected

with the elucidation of the complicated spectrum of the hydrogen molecule. While the types of vibration of the hydrogen atom are comparatively simple and well understood, very little progress had been made in deciphering the spectrum of the molecule, which shows several thousand bands. The interpretation of this spectrum, which is obviously of great importance to the study of molecular structure, presents peculiar difficulties. These have been largely overcome, and Prof. Richardson has taken a large part in this successful work. The results have been interpreted in terms of the wave-mechanics, and the values obtained for the moment of inertia of the molecule, its ionisation potential and heat of dissociation, fit in well with other observational data. The peculiar alternating intensity of the band spectra appears to show that the hydrogen nucleus has a moment of momentum, like the electron.

In addition to a number of papers on this subject Prof. Richardson, in collaboration with his research students, has continued his important investigations in thermionics, ionisation potentials and X-rays ; nor must I omit mention of his excursions into the field of theoretical physics, where he has considered the application of the new mechanics to the theory of the extraction of electrons from a cold conductor by an electric field, and, in conjunction with Mr. Flint, to other problems of theoretical interest.

The first Foulerton Professor, the late Prof. E. H. Starling, was appointed in 1922, and I had occasion last year, in recording his death, to refer to his distinguished record of research during his tenure of the chair. The second Foulerton Professor, Dr. Archibald Vivian Hill, was appointed less than three years ago, in January, 1926. Since then he has carried out an important series of investigations on the energy changes and physico-chemical processes involved in the measurable vital activities of muscle and nerve. He has measured, for instance, with specially devised apparatus of extreme delicacy, the minute quantity of heat liberated in the passage of a wave of excitation along a nerve, and also the different factors concerned in the efficient muscular activity of man, as exhibited in the running of trained athletes. It is significant of the wide interest in these researches, to which his Foulerton Professorship has enabled Dr. Hill to devote his whole time, that they have been published, largely in our own 'Proceedings,' under the names of some sixteen investigators, who have come to work with him from seven different countries.

The Council have recently decided to fill the other Foulerton chair, vacant since the death of Prof. Starling, and I am able to make this first announcement of the appointment thereto of Dr. Edgar Douglas Adrian, a Fellow of our

Society and of Trinity College, Cambridge, and hitherto Lecturer in Physiology in that University. Dr. Adrian has already a distinguished record as an investigator, especially in the physiology of the nervous system. In recent years, with the aid of apparatus using the modern means of electrical amplification, he has been engaged in recording and analysing the minute changes transmitted, from an excited peripheral sense-organ, along the conducting system of the nerves—changes which, on arrival at a nerve-centre in the brain of a conscious being, would result in one or another form of sensation. In this, or in other important fields of investigation, we may wish Dr. Adrian a long and fruitful use of that greater freedom for research which our Royal Society Professorships afford. Dr. Adrian will for the present continue his researches in the Physiology Laboratory, Cambridge.

In my address last year, I referred to recent advances in the production of very high voltages for technical purposes, and the application of these voltages to highly exhausted tubes in order to obtain a copious supply of high-speed electrons and atoms and high-frequency radiation. It is of interest to note how rapidly in recent years our ideas have widened as to the possibilities of production of very high-frequency radiation of the X-ray type, both by artificial and natural processes.

In my address this afternoon, I shall briefly consider the present state of our experimental knowledge on this subject, and the various directions of attack by which we may hope to get further information. On the quantum theory, the energy associated with a quantum of radiation of frequency ν is given by $h\nu$, where h is the well-known constant of Planck. When swift electrons impinge on matter, radiation of an X-ray type is generated over a wide range of frequencies, and it has been verified experimentally that the maximum frequency of the radiation obtainable in this way is limited by the relation $E = h\nu$, where E is the energy of motion of the electron, a result in accordance with energy considerations.

For purposes of discussion, it is very convenient to express the energy of a quantum not in ergs but in terms of a potential difference in volts, through which an electron must fall to acquire an equal energy. Expressed in this way, the energy of a quantum of green light corresponds to 2 electron-volts, or 2 volts for brevity. Before the advent of X-rays, the highest frequencies examined were confined to the ultra-violet part of the light spectrum, corresponding to less than 10 volts. Following the discovery of X-rays and the application of methods for determining their frequency, we have been enabled

to study radiations over a wide range of individual energy, varying from a few hundred volts to 300,000 volts or more. By the use of special gratings and other methods, the gap in frequency between ordinary ultra-violet light and soft X-rays has been bridged in the last few years. There appears to be no limit to the maximum frequency that can be obtained by the bombardment of matter with electrons, except the practical difficulty of obtaining streams of the requisite high-velocity electrons. In some recent experiments in the Institute of Technology, Pasadena, about 1 million volts has been successfully applied for a short time to a suitably designed X-ray tube. It is stated that the X-rays obtained were of such intensity and penetrating power that they could easily be observed by the luminosity on a phosphorescent screen 100 feet away.

So far our experiments in this direction have been limited to about 1 million volts, and we have not yet been able to produce X-rays in the laboratory of penetrating power equal to that shown by the gamma-rays spontaneously emitted by radioactive bodies. The highest frequency observed in their transformations corresponds to between 3 and 4 million volts. Some recent experiments indicate that the gamma-rays which accompany the weak radio-activity of potassium are of still greater penetrating power than the rays from radium, but no definite estimate of the maximum frequency has so far been made.

There is, in addition, another general method of estimating the frequency of radiation that may arise in certain fundamental atomic processes of a simple type. According to modern views energy and mass are closely connected, and the relation between the energy E resident in a mass m is given by the well-known equation of Einstein $E = mc^2$, where c is the velocity of light. According to this view, if any system decreases in mass by internal rearrangement, the total energy lost in the process is given by the product of the change of mass multiplied by c^2 . If this energy is emitted in the form of a radiation of one definite frequency ν , then $h\nu = c^2 dm$, where dm is the accompanying change of mass of the system. On account of the very small change of mass even for a large emission of energy, it is difficult to give a direct experimental proof of this relation, but there seems to be little doubt of its general validity. Even for the radioactive bodies, which in their successive transformations spontaneously emit a very large amount of energy per atom, in the form of alpha-, beta- and gamma-rays, the effect to be expected is small and difficult to measure. The atom of uranium, of mass about 238, after successive transformations involving the loss of eight alpha-particles changes into an isotope of lead, of mass about 206.

It is to be anticipated, that, if the methods of positive ray analysis could be applied to these elements, the difference between the atomic masses of uranium and the resulting lead would include not only the mass of 8 helium nuclei in the free state, but also about 0.05 unit of atomic mass, corresponding to the total emission of energy of about 46 million electron-volts per disintegrating atom of uranium. This difference—about 1 in 4,000—should be just detectable by the methods employed by Aston in his study of isotopes. Similarly the change of mass in each transformation can be deduced if the energy released during the process is known experimentally.

We shall now consider the application of these ideas to certain nuclear processes. It is now generally accepted that the nuclei of all the elements are composed of protons (hydrogen nuclei) and electrons. While it is, of course, difficult to give a definite proof of this hypothesis, we know that it is strongly supported by the work of Aston on the atomic masses of the isotopes of the elements, and by the experiments on the liberation of protons from certain light elements when bombarded by swift alpha-particles. It is generally supposed that the helium nucleus is composed of a close combination of four protons and two electrons. The mass of the helium atom is 4.00216 ($O = 16$), while the mass of four hydrogen atoms in the free state is 4×1.0078 . There is in consequence a loss of mass of 0.029 units in the formation of the helium atom. This indicates a loss of energy of 27 million electron-volts in the process of building a helium nucleus from free protons and electrons. If it be possible to imagine that in some way this energy is emitted catastrophically, in a single quantum of radiation, the energy of the quantum would correspond to 27 million volts. The energy emitted per atom is thus very large, and it has been suggested by Eddington and others that the formation of helium from hydrogen nuclei and electrons may be one of the sources of the energy radiated from the stars.

In a similar way the total energy emitted during the formation of any atom of known mass from free protons and electrons may be estimated. Since the proton in a free state has a mass 1.0073, and a mass about 1.000 in the average nuclear combination, the energy released per proton is about 7 million volts. For example the atomic weight of the most abundant isotope of mercury (atomic number 80) is 200.016, and this presumably contains 200 protons, of mass nearly unity, and 120 electrons. Disregarding the small mass due to the electrons, we may conclude that the total energy emitted during the formation of a mercury atom from free protons and electrons is about 1400 million electron-volts.

When we consider the extreme complication of such a heavy nucleus and the number of its component parts, it is difficult to believe that this emission of energy can take place in one single catastrophic act. It is so much more likely that the energy is emitted in a step by step process during the organisation of the nucleus. Except for light atoms, where the nuclear structure is simple, it is to be expected that the radiation of energy from all complex nuclei would occur in successive stages.

On the other hand, there is one possibility to consider, which was first put forward by Jeans to account for the long lives of the hot stars. He supposes that even the protons and electrons are not indestructible, but may under unknown conditions be transformed into radiation. The total internal energy of the electron is about 500,000 volts, but of the proton 1,840 times greater, or about 940 million volts. If we suppose the proton and electron to disappear together in the form of radiation, there must be an enormous liberation of energy. If this energy be emitted in a single quantum, we should expect to obtain a gamma-radiation corresponding to about 940 million volts. Such an hypothesis is admittedly of a very speculative nature and may be very difficult of direct proof or disproof.

Apart from the radioactive bodies, we have no definite experimental evidence of the emission of penetrating radiations, either in the formation of atoms or destruction of protons, and it may be that the processes considered do not take place under the conditions of our experiments on the earth. On the other hand, the long life of the hot stars indicated by general astronomical evidence does seem to demand some such process or processes, in which the liberation of energy is enormous compared with the mass involved.

It is thus of very great interest to examine whether any direct experimental evidence can be obtained of the existence of such extraordinarily energetic gamma-rays. This interest is heightened by the experiments in recent years which have shown the existence of an extremely penetrating type of radiation, sometimes called the "cosmic" rays, in our atmosphere—a radiation much more penetrating than the gamma-rays from the radioactive bodies. This radiation has been detected and measured by the small ionisation produced in a closed electroscope. The initial observations were made by Hess and by Kolhörster, and we owe much to the admirable experiments of Millikan and Cameron, who have carefully examined the absorption of this radiation by the water of mountain lakes, which are practically devoid of ordinary radioactive matter.

It is clear from these experiments that the radiation is complex in character,

and that there are present radiations which are able to pass through 17 metres of water for a reduction of intensity to one-half value. It is natural to suppose that this radiation is of a gamma-ray type, but it should be borne in mind that the effects so far observed would be equally explicable if the radiations consisted not of high-frequency gamma-rays, but of high-energy electrons entering our atmosphere.

Assuming, however, that the radiation is of the gamma-ray type, it is necessary to consider the factors that determine the absorption of such a radiation by matter. During the past 20 years, the problem of the nature on the absorption of X-rays and gamma-rays by matter has been the subject of detailed investigations, and there is now a general consensus of opinion on the main features of the processes involved. In the case of the heavier elements, the absorption of ordinary X-rays is mainly due to the interaction between the radiation and the electrons in the atom, whereby the energy of the quantum of radiation is transferred to the electron. This is generally known as the "photoelectric" effect. In addition there is a relatively small loss of energy due to the scattering of the incident radiation by the electrons; but in general, except for very high-frequency X-rays and light elements, the absorption due to the photoelectric effect predominates. The case is quite different when we deal with penetrating gamma-rays, where the loss of energy due to the process of scattering becomes relatively much more important, and for radiation of energy of the order of 100 million volts almost completely governs the absorption.

The main features of this scattering, known as the Compton effect, are now well understood. There is an occasional interaction between the quantum of radiation and the electron in an atom, whereby the radiation is scattered and the electron set in motion. The scattered radiation is always of lower frequency than the incident radiation, the difference depending on the angle of scattering. In this type of encounter between radiation and an electron, both momentum and energy are conserved, and consequently the energy given to the electron depends on the nature of the encounter, and thus on the angle of scattering of the radiation. The essential correctness of this theory has been verified by several distinct methods.

When a pure radiation of definite frequency is passed through matter, there always remains some transmitted radiation which has not been transformed, but mixed with it are degraded radiations of much lower frequency and swift electrons set in motion by the process of scattering. The ionisation observed in a closed vessel is probably mainly due to the electrons liberated by scattering in the medium and the walls of the containing vessel.

Assuming that the laws of the Compton process of scattering are valid for high-frequency radiation, there still remains the difficulty of estimating the probability of such scattering encounters, for on this probability depends the actual magnitude of the absorption coefficient. Different methods of calculating this probability have been given by A. H. Compton, Dirac, and recently by Klein and Nishina. The theory of Compton is based mainly on classical analogies, and that of Dirac on the earlier quantum mechanics. Recently the problem has been attacked again by Klein and Nishina ('Nature,' Sept. 15, 1928), using the later relativistic form of wave-mechanics formulated by Dirac. The calculated absorption coefficients for high-frequency radiations differ materially from one another on these three theories, and in particular the theory of Klein and Nishina gives a greater absorption coefficient for a given high-frequency radiation. For radiations of individual energy more than 100 million volts, the coefficient is about five times greater than that given by the formula of Dirac.

Unfortunately the experimental evidence available from a study of the absorption of the most penetrating gamma-rays from radioactive bodies is not complete enough to give a definite test of the validity of these theories. However, Mr. Gray, of the Cavendish Laboratory, who has made a careful examination of existing data on the absorption of gamma-rays, informs me that the evidence as a whole is more in accord with the theory of Klein and Nishina than with the earlier theories of Compton and Dirac. It is evident, however, that in view of the importance of the question, a careful determination is required of the absorption and scattering of gamma-rays, of as definite frequency as possible, in order to distinguish between the various theories.

It is of interest to note that the absorption coefficient of the most penetrating type of radiation, deduced by Millikan and Cameron from their experiments, is in excellent accord with that to be expected on the Klein-Nishina theory for a quantum of energy 940 million volts—the energy demanded for the transformation of the internal energy of the proton into radiation. Although this agreement is suggestive, our theories of absorption are at present too uncertain to place much weight upon it. Even if subsequent experiment should prove the correctness of an absorption formula within a certain range of frequency corresponding to the gamma-rays there would still be the need of extrapolating the formula over a very wide range, say from quantum energies of 3 million volts to 1,000 million volts, to include the ultra-penetrating rays observed in our atmosphere.

In addition there are a number of new factors which may have to be taken

into consideration when we are dealing with the passage of very high-frequency radiation through matter. In the ordinary theories, the scattering of the radiation is supposed to be confined to the extra-nuclear electrons, but if we are dealing with a quantum of energy corresponding to the order of 100 million volts, it is not unlikely that the nuclear electrons may be effective in scattering as well as the outer electrons. Such an effect is to be expected if the energy of the quantum is large compared with the energy required to release an electron from the nucleus. In addition there is always the possibility, and even the probability, that such energetic radiations or the swift electrons liberated by them may be able occasionally to disintegrate the nucleus of the atom in their path.

For all these reasons, it is evident that much more information is required before we can draw any but tentative conclusions as to the nature of the penetrating radiations in our atmosphere. So far, experiments have been mainly confined to measuring the ionisation produced in a sealed electroscope. Further experiments are required, which will give us definite indication of the energy of the swift electrons present in the atmosphere, for this will give us valuable information on the maximum frequency of the radiation present, quite independently of the exact accuracy of our theories of absorption.

Continued observations made in a Wilson expansion chamber should throw much light on the nature of the particles which produce the ionisation in a closed vessel, and with the addition of a magnetic field of sufficient intensity the curvature of the tracks of beta-rays should enable us to determine their individual energy. Experiments of an analogous kind have already been made with an expansion chamber by Skobelzyn, in order to determine the relative intensities of the main gamma-rays emitted by radium C. In the course of these experiments he has observed on several occasions the trails of very energetic beta-particles, probably arising from the ultra-penetrating radiation in our atmosphere.

During the present year Prof. Hans Geiger has developed a modified form of beta-ray counter, which records each beta-particle entering a vessel of considerable volume in any direction. This new method is so delicate that it may prove very useful in counting and even recording the number of beta-particles produced by the penetrating radiation. While it is to be hoped that in the years to come we may have available for study in our laboratories swifter beta-rays and higher-frequency radiation than we have to-day, we can hardly hope in the near future to produce artificially radiations, atoms and electrons

which have an individual energy of the order of 100 million to 1000 million volts, such as are present in our atmosphere.

It is thus of great interest and importance to use every promising method of attack to throw light on the nature and origin of these penetrating radiations and the effects arising in their transmission through matter. The magnitude of the effects to be observed is small and not easy to measure with accuracy; but with the ever-increasing delicacy of methods of attack we may hope to gain much further information. The study of these extraordinarily penetrating radiations is not only of great interest in itself, but also for its promise of throwing new light on fundamental processes in our universe connected with the building up and destruction of atoms. It may take many years of faithful experiment before the evidence is sufficient to test the correctness of the numerous interesting speculations that have been advanced to account for the origin and nature of these radiations.

We now pass to the presentation of the medals.

The Copley Medal is awarded to Sir Charles Parsons, O.M., F.R.S.

In the world of mechanical engineering the genius of Charles Parsons has opened up a new era. He has originated and developed a new type of thermal engine entirely flexible and adaptable, and capable of high efficiency, combined with concentration of power never even imagined before.

By continuous practical effort for the past 45 years, aided by remarkable mathematical insight acquired in his University days, he has perfected the parallel flow compound steam turbine, and has applied it successfully to electric generation and to marine propulsion, both attaining to an unprecedented scale. In this progress there have been involved great ingenuity in practical design and careful research into the dynamics of the flow of steam between fixed and moving blades, and the scientific determination of the best proportions for the successive stages of expansion and the degrees of superheating. The result has been that while the utilisation of heat in the best triple-expansion reciprocating steam engine amounts to 17 per cent. of the whole, the Parson's large central station turbines now convert 25 per cent. into mechanical power, and in still larger turbines 28 per cent. is anticipated.

The first steam turbine of 4 kilowatts was used in 1885 for electric lighting. This was followed by the development of turbines of increasing power, reaching 5,000 kilowatts in 1910. At present turbines of 20,000 and 30,000 kilowatts are in operation.

The application to marine propulsion was signalised in 1897 by the appearance

of the "Turbinia," a small experimental craft of 200 tons, developing the extraordinary speed of 33 knots. Large turbine-driven destroyers for the Navy rapidly followed. In 1904 the cruiser "Amethyst," of 3,000 tons, was propelled by Parsons turbines of 14,000 h.p., with success so conspicuous that all new warships of all classes were fitted with steam turbines, so that by 1912 the Royal Navy had 7 million horse-power of Parsons turbines in operation. In the merchant navy progress was equally rapid. The first turbine vessel was the Cunard liner "Carmania," and now all large high-speed liners, such as the "Aquitania," are turbine-driven.

During this remarkable development numerous problems arose, urgently demanding a solution, involving a precise study of jet velocities, leakage, turbulent flow and vacuum augmenters. The phenomena involving cavitation of screw propellers opened up new fields, of abstract as well as practical interest.

Naturally other eminent inventors have taken a share in this progress, but they would doubtless all concur that Sir Charles Parsons has been greater in the scientific development of thermal power produced by steam than any engineer since James Watt.

A recent side-product of Sir Charles Parsons' activities, here stimulated by heredity, has been the revival of the British scientific industry, once conspicuous, of optical glass and telescopic construction, while some of his hours of relaxation have been spent in the strenuous endeavour to crystallise carbon into diamonds by catastrophic processes.

The Rumford Medal is awarded to Prof. Friedrich Paschen.

Prof. Paschen is especially distinguished for his important contributions to spectroscopy. He early acquired remarkable skill in the investigation of infra-red radiation and made valuable determinations of the distribution of energy in the spectrum of a black body, giving the first experimental proof of the law that the frequency of maximum energy is proportional to the absolute temperature. He afterwards made numerous observations of the infra-red emission spectra of various elements, which were of fundamental importance for the development of our knowledge of series in spectra, and subsequently for the theory of spectra in relation to atomic structure.

Prof. Paschen has also contributed in a notable degree to the precise measurement and series classification of spectrum lines in general. His masterly analysis of the highly complicated spectrum of neon affords an admirable example of his insight and skill. His work on the spectrum of doubly-ionised

aluminium also stands out conspicuously as giving the first proof of Bohr's deduction that the series in such a spectrum should be characterised by a constant nine times greater than the Rydberg constant which is applicable to the spectra of neutral atoms. Another striking contribution was made by his extremely delicate observations of the fine structure of the lines of ionised helium, which proved to be in close agreement with Sommerfeld's theory, and led to one of the most trustworthy estimates of the mass of an electron. Prof. Paschen has also long been one of the foremost workers on the Zeeman effect, and the results which he has obtained, including the discovery of the well-known Paschen-Back effect, have been invaluable for theoretical discussions.

In all his investigations Prof. Paschen has shown extraordinary skill in the design and manipulation of apparatus, and the whole of his work is characterised by an obvious striving for the greatest attainable precision.

A Royal Medal is awarded to Prof. Arthur Stanley Eddington, F.R.S.

The contributions to knowledge of Prof. Eddington within the past ten years have been mainly in connection with the internal constitution of stars and with the generalised theory of relativity.

By an examination of the conditions of equilibrium of a typical giant star he showed that these were more easily satisfied when account was taken of radiation pressure. His results were somewhat modified when the extent of the ionisation at the high temperature in the interior of a star was pointed out by Jeans and Newall. He formulated a complete theory of the internal structure of a star, assumed to be a non-rotating whirl of atoms and electrons, with radiation gradually forcing its way to the surface; further, he pointed out that the masses of stars, which are found by observation not to vary greatly, ranged about the point where radiation pressure balances gravitation. Later, Eddington obtained a theoretical relation between the mass and absolute luminosity of giant stars. Taking Capella as a starting point, he found agreement with his theoretical results not only among giant stars but also among dwarfs, which had hitherto been supposed to have a liquid or solid nucleus; and he explained this result as due to the closer packing that is possible when atoms are stripped of their outer electrons. The companion of Sirius is the extreme example of this condition.

Eddington has also worked out a mathematical theory of Cepheid variables on the assumption that they are oscillating radially. These extremely bright stars play a fundamental part in several astronomical questions, and

it is of the greatest importance that their physical condition should be investigated.

In connection with the theory of relativity, he conducted in 1919 one of the two eclipse expeditions which verified the deflection of light rays from stars near the sun. He also developed the theory, to a certain extent on the philosophical side, but considerably on the analytical side, especially with regard to the electromagnetic and gravitational fields.

Prof. Eddington has attained international fame by the brilliance of his contributions to astronomical science.

A Royal Medal is awarded to Dr. Robert Broom, F.R.S.

During the course of thirty-three years' search in Australia and South Africa Dr. Broom has made a very large number of important discoveries in vertebrate palæontology, embryology and morphology that shed new light upon the problems of the origin of mammals, lizards, crocodiles and birds, the significance of which has been interpreted in his Croonian Lecture ('Phil. Trans.,' 1914) and 285 memoirs. His researches represent the most significant contribution made by any one investigator to the determination of the relationships of the main groups of vertebrate animals, and to the definition and solution of the problems involved in the evolution of the higher groups.

At the time when he first went to South Africa, 35 genera and 65 species of fossil reptiles had been identified in the Karoo beds; very little was known of their structure, and the classification was in a state of apparently hopeless confusion. He tripled the number of known genera and quadrupled the number of species. He worked out the details of the anatomy of most of the groups, and established an orderly classification which has now been universally accepted by the scientific world. He is now actively engaged in the investigation of some of the outstanding problems involved in the evolution of mammals and birds.

The Davy Medal is awarded to Prof. Frederick George Donnan, F.R.S.

Prof. Donnan is, like his master van't Hoff, a man of ideas. Early in his scientific career he wrote on the nature of soap emulsions and on the theory of capillarity and colloidal solutions. Being thus engaged with problems of surface activity, and being also intimately conversant with the electrolytic dissociation hypothesis, he put two and two together and made thereby not four, but a multitude—the mark of original scientific genius. His theory of membrane equilibrium and membrane potential is an

achievement of the first rank, and has been the starting-point of numerous studies not only in the domain of pure chemistry, but more especially in biochemistry, where the conditions for displaying the phenomena he predicted are often encountered. Physiological chemists have seized on his idea and utilised it in many fields of investigation.

Donnan's researches on surface tension and adsorption at liquid-liquid interfaces have led to results of the greatest interest, and his verification by means of nonylic acid of the Gibbs' adsorption formula is a most brilliant experimental conception. A by-product of Donnan's activities during the War is a theory of the action of gas-scrubbers, based on the velocity of absorption of gases by liquids.

Not only has Donnan by his own researches done the highest service to his science—he has founded a school of physical chemistry, whence issues a steady flow of admirable researches, which he has suggested, and of young physical chemists, whom he has inspired.

The Darwin Medal is awarded to Dr. Leonard Cockayne, F.R.S.

The award of a Darwin medal to Dr. Cockayne is fitting because of the distinction of his work in fields in which Charles Darwin himself laboured. That distinction has been gained by the use of the Darwinian method: a true naturalist, Dr. Cockayne has waited patiently upon facts before drawing conclusions. For over thirty years he has made it his task to deepen and widen our knowledge of New Zealand botany in the broadest sense. He has not only worked himself with outstanding ability and untiring energy; he has imparted his enthusiasm to a school of younger colleagues, eager to advance his labours on the lines he has laid down. The excellence of Dr. Cockayne's work, from the ecological point of view, is recognised by botanists in every country and has made him one of the foremost living students of plant-association; the taxonomic studies rendered necessary by his ecological results have led to those remarkable discoveries of natural hybrids in New Zealand that have won for him a world-wide reputation, and have made on modern thought an impression akin to that produced by the results of Mr. Darwin's studies of plants under domestication. Dr. Cockayne's researches have had, on sylvicultural and agricultural procedure, a practical bearing which has been appreciated by, and has influenced the policy of, New Zealand statesmen.

The combination of philosophic outlook and lucid exposition that marks his contributions to natural knowledge, may also explain the invitation, accepted by Dr. Cockayne, to contribute a monograph on the flora of New Zealand to

'Die Vegetation der Erde,' edited by Dr. Engler—a compliment no other British botanist has been paid. It does at least account for the remarkable local effect of Dr. Cockayne's book 'New Zealand Plants and their Story.' An eminent Continental ecologist, well acquainted with the philosophic importance of Dr. Cockayne's labours, has said "it is wonderful how Cockayne has succeeded in interesting the population of a new country in botany."

The Sylvester Medal is awarded to Prof. William Henry Young, F.R.S.

Dr. W. H. Young has taken a very prominent part in the development of the modern theory of functions of real variables, and in its application to the theory of Fourier's and other series. During the present century he has published an immense number of memoirs and notes dealing with this branch of mathematics and containing important advances in the subject; many of these are strikingly original, and even the smaller of them throw light on some particular points. His earlier work dealt chiefly with the theory of sets of points, and contains important developments on the lines laid down by G. Cantor and Harnack. He soon proceeded to apply this theory in the integral calculus, and he obtained a general definition of the integral which is essentially equivalent, although somewhat less simple in form, to that given, about the same time, by H. Lebesgue, which latter has become a corner stone of modern analysis. Much of Dr. Young's work has also proved to be a starting point for further investigations by other mathematicians. Instances of this feature in his work are his generalisations of the theorems of Parseval and Riesz-Fischer in the theory of Fourier's series; his investigations on the properties of Fourier's constants and of the series conjugate to Fourier's series. By means of his conception of restricted Fourier's series he was enabled to devise a method by which conditions of convergence, summability, &c., known to hold good for Fourier's series, could be carried over to series of Legendre's and Bessel's functions. Many other investigations in the domain of integration and Fourier's series, too numerous to be mentioned in detail, have contributed notably to our present knowledge in this department of analysis.

The Hughes Medal is awarded to M. Le Duc de Broglie.

Maurice François César, Duc de Broglie, member of the Academy of Sciences, is distinguished especially for his pioneer researches on X-ray spectra and secondary beta-rays. He was one of the first to obtain the complete emission

spectrum of X-rays and to study X-ray absorption spectra, while his work on the magnetic spectrum of the beta-rays, arising from the passage of X-rays through matter, has proved of great importance. He founded in Paris a private laboratory directed by him, which is devoted to researches on X-rays and allied subjects. An experimenter of unusual skill, he and his co-workers have made important contributions to knowledge in many fields.

The Respiratory Quotient of the Excess Metabolism of Exercise.

By C. H. BEST (Toronto), K. FURUSAWA (London), and J. H. RIDOUT (Toronto).

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INTRODUCTION.

In recent years much controversy has been aroused as to whether the body uses carbohydrate alone in order to provide energy for the recovery process from muscular exercise, or whether the other foodstuffs can be used directly for this purpose. One of us (Furusawa), from a study of the respiratory quotient of the excess metabolism, concluded that carbohydrate is alone responsible for supplying the energy for short-lived exercise, while other foodstuffs must be converted into carbohydrate before they are so used by muscle (1). This conclusion has been contested by several workers and no means of reconciling the different observations was apparent.

A new factor, however, has recently been discovered, in the intensity of the exercise undertaken. This may help to explain the discordant results of the various workers on this subject. During the spring of 1927 Furusawa, Hill and Parkinson (2) made a large number of observations on the gaseous exchange of "sprint running," in order to determine the "mechanical efficiency" of this form of very severe exercise. Incidentally the results revealed a most remarkable phenomenon in respect of the respiratory quotient of the excess metabolism resulting from such exercise. Briefly stated, the excess respiratory quotient showed a wide divergency from unity (1.2 to

1.6). It has often been observed that the respiratory quotient rises above unity during or immediately after severe exercise. The assumption of the "blowing off" of CO_2 would adequately meet these cases. On the other hand, the high "excess" respiratory quotient, which we will now describe, has never been described before. In fact, however, this new phenomenon appeared invariably, and as it seems now most conspicuously, in many experiments, before its existence was recognised.

The results described in the present paper were obtained in three stages, following the discovery of the above-mentioned phenomenon. Prof. A. V. Hill suggested to two of the present authors (Best and Ridout) to make similar observations independently. Accordingly a large number of experiments were performed in Toronto during the period from June, 1927, to February, 1928, in which the whole range of severity of exercise was covered. Moreover a few observations on blood sugar after severe exercise were recorded. In the course of these experiments it was invariably found, contrary to the report made by Furusawa in 1925, that with very moderate exercise the respiratory quotient of the excess metabolism does not reach unity, but remains at a low level. To find the cause of this discrepancy, further observations in this region of exercise were made in London by Furusawa, during May and June, 1928. Before going further into a description of the experimental results, a few words on the respiratory quotient and on the precautions required for this kind of experimental work are required.

The respiratory quotient at a given moment is nothing but the ratio of the CO_2 being expired to the oxygen being absorbed by the respiratory organs at the moment in question. It does not, except in special circumstances, indicate the nature of the contemporary combustion of foodstuffs inside the body. It tells only a partial history of what is happening, and unless the whole history is known we cannot draw any conclusion, from a single observation of the respiratory quotient, as to the nature of the oxidation. In a recent paper Cathcart and Markowitz (3) have discussed the question of the respiratory quotient. They administered equivalent quantities of glucose, sucrose, galactose, l  vulose and dihydroxyacetone to a normal subject. With all the substances employed, except glucose, the respiratory quotient responded immediately after the administration, by a sharp rise over unity, whereas glucose caused only a gradual increase. This extraordinary result could not be explained by the conception of the respiratory quotient as a combustion quotient. Cathcart and Markowitz suggested that the non-protein respiratory quotient represents, not a combustion quotient, but the algebraic sum of (1)

the transformation carbohydrate \rightleftharpoons fat, and (2) the oxidation of carbohydrate for the liberation of muscular energy.

In connection with the measurement of the gaseous metabolism associated with exercise and recovery, we feel that it is necessary to point out that the excess metabolism is estimated as a difference between two large quantities, and that very stringent precautions must be taken before results can be regarded as significant. These have not always been taken, and their neglect may render observations worthless. Such precautions are :—

(1) *Fore-period of Rest.*—This should not be less than 30 minutes before the first resting collection is taken ; this allows time for the recovery process to be completed after the ordinary exercise of daily life in, or coming to, the laboratory. If harder exercise has been undertaken the fore-period should be prolonged. Mouth-pieces, or similar apparatus used in the experiment, should be worn during this period so as to avoid any possible change of respiratory movement when the apparatus is first put on.

(2) *Collection of Expired Air during and in Recovery from Exercise.*—It is advisable to collect the whole expired air in one bag or suitable container. In cases where the collection is made in several portions care must be taken during the change of apparatus to avoid any escape of the expired air. All the expired air during exercise and recovery must be collected : it is little use to make sporadic collections and to attempt to interpolate the region between them.

(3) *Time Allowed for the Recovery Process.*—The time required for recovery is dependent upon the severity of exercise. Even when the exercise is of very short duration, it may, if it be sufficiently violent, require a considerable period of recovery. For example, a 100 yards run at top speed (we mean really top speed—the absolute maximum speed that the subject can attain) may require more than 30 minutes for recovery, even in subjects who are able-bodied and in comparatively good training. The period necessary for recovery cannot be guessed, it must be determined by experiment. Unless recovery is known to be complete the respiratory quotients have no meaning, except in so far as they show the production and removal of acid in the body.

(4) *Gas Analysis.*—It is necessary that the apparatus used for the gas analysis, and the skill of the experimenter, should be such that the analyses are accurate to 0.01 per cent., since large amounts of gas have to be dealt with and comparatively large quantities to be subtracted from each other. It is advisable to test the apparatus on air each day. It is inexpedient to use oxygen mixtures other than air for this type of experiment, since a small error

in measurement, or the lack of equilibrium of gases in the body, may lead to fallacious results, as pointed out previously by one of us (4).

(5) *Subject of Experiment.*—Even when all precautions are taken and the difficulties connected with accurate analysis, etc., overcome, the success of the experiment depends upon the choice of a practised and suitable subject. The subject must be acquainted with the apparatus used and must approach the experiment with nonchalance. Self-consciousness, or awareness of the apparatus, unpleasant sensations in mouth, nose and respiratory passages associated with its use, and similar circumstances, may change the rate and depth of breathing, or, altering the state of attention and muscular tone of the subject, may change his metabolism. To employ a subject showing irregular results is not to prove that *any* foodstuffs may be used for the metabolism associated with exercise: such results in our opinion are a sign merely of insufficient experimental precautions.

In this connection Table I shows the important rôle played by the subject. The subject here was an employee of University College, London, and was

Table I.—Illustrating inconsistency of Results with unsuitable subject (London).

Exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.
	c.c. per min.	c.c. per min.	c.c.	c.c.
1.—270 steps per min. for 30 secs. The subject failed to maintain this speed.	O ₂ ... 352	337	18373	2871
	CO ₂ ... 316	325	16566	2144
	R.Q. ... 0·89	0·96	0·89	0·75
	F.P. ... 30 mins.		T.C. 45 mins.	
	Vent. ... 11·37	10·75		
2.—30 secs. as hard as the subject could go.	O ₂ ... 366	392	19956	2143
	CO ₂ ... 309	370	17976	2020
	R.Q. ... 0·84	0·94	0·90	0·94
	Vent. ... 11·3	12·1	T.C. 47 mins.	
	F.P. ... 50 mins. after a previous run			
3.—40 secs. as hard as the subject could go.	O ₂ ... 378	344	19599	3715
	CO ₂ ... 276	261	19599	7785
	R.Q. ... 0·73	0·75	1·00	2·09
	F.P. ... 50 mins. after a previous run	9·4	T.C. 44 mins.	

Exercise—standing running in each case.

F.P. = foreperiod of rest.

T.C. = time of collection.

R.Q. = respiratory quotient.

Vent. = ventilation in litres per minute.

once engaged for a short period for experiments on the oxygen requirement of barrow work. He is acquainted, therefore, with the kind of experiment performed and is not particularly concerned at being asked to place a mouth-piece in his mouth and to breathe into a bag. We hoped on him to get consistent results for comparison with those on our other subjects. Three successive experiments (and others not recorded here) were so obviously inconsistent, as is shown in the table, that no more were performed on him.

THE HIGH RESPIRATORY QUOTIENT OF THE EXCESS METABOLISM DUE TO
RUNNING AT TOP SPEED FOR A SHORT PERIOD.

Table II shows results obtained at Cornell University, Ithaca, N.Y., during the spring of 1927. The experiments were conducted mostly on the open track at the athletic ground, but occasionally inside the drill hall of the University. In the first 11 experiments a 60 yards run and 10 yards pull up was the exercise performed: in the later experiments a longer distance was covered. The subject rested before the experiment at the end of the track, or during very cold weather inside the pavilion adjoining it, and after a suitable resting period the first resting collection was made in a rubber-fabric bag of the usual type. It is possible to run 100 yards without breathing, and after practice even 150 yards. This makes the experiment simple. The mouth-piece, nose-clip and bag were kept on the end of the track, in such a way that they could be employed immediately the subject reached it. By this method it is possible to avoid the interference with bodily movement which results from the use of apparatus during the run. Occasionally in the longer runs the subject was unable to hold his breath the whole way and one or two breaths were taken, involving the loss of some 4 to 8 litres of expired air. Compared with the large amount expired up to the end of recovery in the case of such long runs this 8 litres is practically negligible and we have neglected it. Experiment No. 13 was made inside the drill hall. In this a cubical bag was carried on the shoulders and the expired air was collected during the entire run. Owing to the time occupied in turning, the duration of the runs in Experiment No. 13 was rather greater, and it was necessary to avoid any loss of expired air such as would have occurred if an attempt had been made to hold the breath till the end of the run. Owing to the bag the speed was reduced considerably in these indoor experiments, as is shown by the times quoted in the Table.

All the earlier experiments of this series were carried out for another purpose (2), namely, to determine the absolute energy expended in a short interval

Table II.—Sprint Running at absolutely Top Speed : Observations made at Ithaca, New York.

Subject : exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.
	c.c. per min.	c.c. per min.	c.c.	c.c.
2.—K.F., 60 yds. run, 10 yds. pull up.	O ₂ . . . 261 CO ₂ . . . 241 R.Q. . . . 0.93 F.P. . . . 20 mins.	255 208 0.82	11080 11435 1.03 T.C. 32.6 mins.	2670 4117 1.54
3.—K.F., ditto	O ₂ . . . 200 CO ₂ . . . 134 R.Q. . . . 0.67 F.P. . . . 20 mins.	199 169 0.85	8870 8738 0.98 T.C. 30.7 mins.	2746 4087 1.48
4.—K.F., ditto	O ₂ . . . 207 CO ₂ . . . 173 R.Q. . . . 0.84 F.P. . . . 20 mins.	203 181 0.89	8692 8846 1.01 T.C. 30 mins.	2542 3536 1.38
5.—J.L.P., ditto	O ₂ . . . 223 CO ₂ . . . 190 R.Q. . . . 0.85 F.P. . . . 26 mins.	234 183 0.78	10068 11145 1.10 T.C. 29.5 mins.	3328 5614 1.68
6.—K.F., ditto	O ₂ . . . 184 CO ₂ . . . 157 R.Q. . . . 0.85 F.P. . . . 30 mins.	206 183 0.89	8815 9120 1.03 T.C. 31 mins.	2770 3850 1.39
7.—K.F., ditto	O ₂ . . . 187 CO ₂ . . . 151 R.Q. . . . 0.81 F.P. . . . 30 mins.	200 153 0.76	8446 7941 0.94 T.C. 30 mins.	2641 3381 1.28
8.—J.L.P., ditto	O ₂ . . . 269 CO ₂ . . . 215 R.Q. . . . 0.80 F.P. . . . 30 mins.	272 202 0.75	9574 10064 1.05 T.C. 22.1 mins.	3596 5457 1.51
9.—K.F., ditto	O ₂ . . . 193 CO ₂ . . . 131 R.Q. . . . 0.68 F.P. . . . 20 mins.	193 130 0.67	6597 6940 1.05 T.C. 21 mins.	2544 4200 1.65
10.—J.L.P., ditto	O ₂ . . . 235 CO ₂ . . . 200 R.Q. . . . 0.85 F.P. . . . 20 mins.	249 206 0.83	8596 9547 1.11 T.C. 20 mins.	3756 5487 1.46
11A.—A.V.H., ditto	O ₂ . . . 290 CO ₂ . . . 229 R.Q. . . . 0.79 F.P. . . . 30 mins.	290 222 0.77	13157 12780 0.97 T.C. 30 mins.	4457 6015 1.34
11B.—A.V.H., ditto, follow- ing 11A.	O ₂ . . . 290 CO ₂ . . . 222 R.Q. . . . 0.77	298 227 0.76	12710 12008 0.94 T.C. 30 mins.	3890 5273 1.35
12A.—A.V.H., 100 yds. run, 20 yds. pull up.	O ₂ . . . 280 CO ₂ . . . 242 R.Q. . . . 0.86 F.P. . . . 30 mins.	286 210 0.73	14020 15714 1.12 T.C. 30 mins.	5530 8934 1.61

Table II--continued.

Subject : exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.
	c.c. per min.	c.c. per min.	c.c.	c.c.
12B.--A.V.H., ditto, follow- ing 12A.	O ₂ 286 CO ₂ 210 R.Q. 0.73	289 210 0.73	14094 14913 1.06 T.C. 30 mins.	5469 8613 1.57
13A.--A.V.H., 220 yds. in 37 secs. to and fro in Drill Hall.	O ₂ 267 CO ₂ 225 R.Q. 0.85 F.P. 30 mins.	266 211 0.79	11902 12526 1.05 T.C. 25 mins.	5240 7076 1.35
13B.--A.V.H., 95 yds. in 14.3 secs. ditto, following 13A.	O ₂ 266 CO ₂ 211 R.Q. 0.79	266 200 0.75	11690 12390 1.06 T.C. 25 mins.	5040 7253 1.43
13C.--A.V.H., 200 yds. in 39 secs. ditto, following 13B.	O ₂ 266 CO ₂ 200 R.Q. 0.75	263 207 0.79	12257 11775 0.94 T.C. 25 mins.	5645 6688 1.18
14A.--A.V.H., 150 yds. run, 20 yds. pull up.	O ₂ 251 CO ₂ ... 212 R.Q. 0.85 F.P. 30 mins.	274 187 0.68	15057 16311 1.08 T.C. 30 mins.	7182 10326 1.43
14B.--A.V.H., ditto, follow- ing 14A.	O ₂ 274 CO ₂ 187 R.Q. 0.68	283 204 0.72	15580 16689 1.07 T.C. 30 mins.	7225 10824 1.48
15A.--A.V.H., 100 yds. run	O ₂ 301 CO ₂ 244 R.Q. 0.81 F.P. 40 mins.	282 219 0.78	14904 14648 0.98 T.C. 35 mins.	4702 6546 1.39
15B.--A.V.H., ditto, follow- ing 15A.	O ₂ 282 CO ₂ 219 R.Q. 0.78	293 213 0.73	15415 15256 0.99 T.C. 35 mins.	5353 7696 1.43
16A.--K.F., 100 yds. run	O ₂ 248 CO ₂ 207 R.Q. 0.83 F.P. ... 30 mins.	240 200 0.83	14455 13694 0.95 T.C. 45 mins.	3475 4537 1.30
16B.--K.F., ditto, following 16A.	O ₂ 240 CO ₂ ... 200 R.Q. 0.83	242 223 0.92	14551 13973 0.94 T.C. 45 mins.	3706 4456 1.20
17.--A.V.H., 150 yds. run	O ₂ ... 300 CO ₂ ... 247 R.Q. ... 0.82 F.P. ... 43 mins.	291 220 0.76	21738 21729 1.00 T.C. 51 mins.	6668 9821 1.47

of running at top speed, and were not expected to throw any light on the respiratory quotient. We found uniformly, however, a very high respiratory quotient for the excess metabolism after such severe exercise, and followed the matter up in the remaining experiments which were performed specially for the object of studying the nature of the metabolism resulting from such efforts. Over the long periods, 20 to 50 minutes, in which metabolism was measured after the run, a respiratory quotient of the *total* metabolism was found equal approximately to unity. Consequently in the last column an unusually high value of the respiratory quotient of the *excess* metabolism is obtained. One of us found in the earlier investigation that the respiratory quotient of the excess metabolism following a short period of exercise is unity.

The apparently anomalous result obtained in this series of observations on running at top speed might at first be attributed to one or other of two causes : firstly, that excessive CO_2 is blown out, owing to the large amount of lactic acid liberated in the very violent exercise of running, and that the recovery process is incomplete in the interval considered ; or secondly, that the resting metabolism has been changed as the result of violent exercise, and more carbohydrate used during the basal oxidations, proceeding synchronously with the recovery process, than during the same oxidations before exercise. The second suggestion amounts in fact to the notion that the extreme violence of the effort has somehow altered the metabolic conditions in the body, so that the whole of the metabolism, and not only that part which is due to exercise, is now carried on at the expense of carbohydrate, for a period approximating to that of the complete recovery process.

If the first suggestion were correct, namely, that CO_2 has been blown off by excessive ventilation and the presence of acid in the body, we should expect a CO_2 retention in the later period of recovery (see Hill, Long and Lupton (5).) This should lead to a lower respiratory quotient in what has been taken as the second resting determination. There is some evidence of a lower final respiratory quotient in experiments 2, 5, 7, 8, 12A, 13A, 13B, 14A, 15A, 15B and 17. In these cases, however, the quantity of CO_2 still being retained (on this hypothesis) is far too small to compensate materially for the large quantity which has been expired in excess, assuming that the respiratory quotient of the excess metabolism is actually unity. Moreover, in the other experiments, the respiratory quotient in the second resting period is equal to or higher than that of the first. In the 17 experiments of Table II, where the fore-period was 30 minutes or more (these are the more reliable), the respiratory quotient of the resting metabolism was 0.80 on the average before the exercise and 0.773 after

the exercise. We are dealing in these cases with an excess of several litres of CO_2 , and the small fall observed on the average in the resting respiratory quotient would allow only 6 c.c. of CO_2 to be retained per minute; so that several hours would be necessary to allow for the retention of the extra CO_2 which has to be accounted for.

There is no reason to suppose that recovery from exercise of whatever severity, lasting only for 10 or 15 seconds, takes a number of hours, and moreover in our observations the oxygen consumption appears to return almost exactly to its original level, showing that recovery is indeed complete. In the whole series of experiments shown in Table II the oxygen consumption in the second resting period is only 3 c.c. on the average greater than in the first period, a matter of just 1 per cent., which shows that to all intents and purposes the recovery process was finished. It would seem impossible, therefore, to regard the excess CO_2 as being due to a washing-out process. To make this point clear, however, in several experiments (Nos. 11 to 16) two or more observations were made on the same subject in immediate succession. It seemed just conceivable that CO_2 might be washed out in a single experiment, that is in the recovery from a single run, but quite impossible that the same thing should happen again and again after a second and a third run. There is a limit to the amount of CO_2 which can be washed out from the body, and since the runs were consecutive the excess of CO_2 would be additive.

A preliminary run was performed with no collection made, so as to get the body of the subject into approximately a steady state of alternating exercise and recovery. This primary run was followed by 30 or 40 minutes of rest and the first observation was then made, consisting as usual of a resting reading, an exercise plus recovery reading, and a second resting reading. After the latter observation was completed another run was carried out and again a collection was made over the recovery period and for a final resting period. Thus two observations were made under exactly similar conditions, having been preceded by a bout of exercise and recovery exactly similar in character to those of the experimental periods. The results were quite consistent: excess CO_2 was found after the second run and after the third one. It seems inconceivable, therefore, that the extra CO_2 is due to excessive respiration and acid formation. Taking for example experiment 12, there is an extra 3.4 litres of CO_2 to account for in the first observation and an extra 3.1 litres in the second observation: presumably the first run, the metabolism of which was not recorded, had the same amount of extra CO_2 associated with the recovery from it. These together, therefore, make an excess of about 10 litres of CO_2 to account for, nearly

half a gram molecule, the equivalent—as an acid—of about 40 gms. of lactic acid. This amount of lactic acid, in addition to that normally present in the tissue at rest, would cause a rise of concentration in the blood and the soft tissues of the body to more than 110 mgm. per cent., a value which is about the maximum ever attained immediately after severe exercise. It seems, therefore, that the excess CO_2 must be accounted for as due to some metabolic change and not by a shift in the acid-base equilibrium of blood and tissues; the quantities involved are much too large for any explanations on such lines.

THE EFFECT OF STANDING-RUNNING AT EXTREME SPEED ON THE RESPIRATORY QUOTIENT OF THE EXCESS METABOLISM.

If the new phenomenon described above could be found for the case of standing-running in the laboratory, it would be much more convenient for investigation, so Furusawa conducted a series of observations on returning to London, for the purpose of further investigating the nature of this excessive CO_2 production. Altogether 10 experiments were made, involving the exercise of standing-running at a speed of more than 260 steps per minute. This exercise was continued only for a short time and was of the utmost severity that the subject could undertake. The results are given in Table III. In order to make as certain as possible of complete recovery 40 to 60 minutes were allowed for the collection of the expired air after exercise. Here again it may be noted that the respiratory quotient of the *total* metabolism over the particular interval studied was about unity. This, of course, is a matter of chance, and with a longer or a shorter interval for collection it is bound to fall or to rise, since the ordinary resting metabolism is attained after 30 to 40 minutes. The *excess* metabolism, as before, has a respiratory quotient distinctly higher than unity, namely, from 1.19 to 1.68. Thus, even in the case of standing running in the laboratory (which cannot be made as severe as track running) this supposed metabolic change can be produced if the exercise be of sufficient violence.

Further experiments were done at Toronto (Best and Ridout), which reconfirmed this remarkable phenomenon. Table IV shows the results obtained with standing-running as fast as possible (except the one at 200 steps per minute). The severity of exercise can be judged from the oxygen required per minute to maintain the exercise; it reached more than 16 litres. The time allowed for the recovery was in every case 50 minutes approximately.

The question naturally arises why this effect was not discovered before,

Table III. —Standing-Running at Extreme Speed: Subject K.F. (London).

Exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism
steps per min.	c.c. per min.	c.c. per min.	c.c.	c.c.
1.—260 for 38 secs.	O ₂ 215 CO ₂ 196 R.Q. 0·91 F.P. 30 mins.	217 207 0·95	18883 19347 1·02 T.C. 60·5 mins.	5815 7157 1·23
2.—270 for 40 secs.	O ₂ 213 CO ₂ 176 R.Q. 0·83 F.P. 30 mins.	229 186 0·81	13849 14784 1·06 T.C. 40 mins.	5009 7544 1·50
3.—270 for 30 secs.	O ₂ 224 CO ₂ 195 R.Q. 0·86 F.P. 30 mins.	235 203 0·86	18549 18737 1·01 T.C. 60 mins.	4779 6797 1·42
4.—270 for 37 secs.	O ₂ 196 CO ₂ 152 R.Q. 0·77 F.P. 45 mins.	197 145 0·73	17808 17516 0·98 T.C. 60 mins.	6018 8606 1·43
5.—276 for 36 secs.	O ₂ 232 CO ₂ 213 R.Q. 0·91 F.P. 45 mins.	228 202 0·88	14196 14908 1·05 T.C. 45 mins.	3846 5571 1·44
6.—276 for 30 secs.	O ₂ 252 CO ₂ 236 R.Q. 0·93 F.P. 30 mins.	242 223 0·92	18490 18662 1·01 T.C. 60 mins.	3670 4892 1·33
7.—276 for 32 secs.	O ₂ 208 CO ₂ 171 R.Q. 0·82 F.P. 32 mins.	203 169 0·83	14206 13617 0·95 T.C. 44·5 mins.	5062 6052 1·19
8.—276 for 39 secs.	O ₂ 209 CO ₂ 171 R.Q. 0·81 F.P. 35 mins.	212 176 0·83	14890 14110 0·95 T.C. 50 mins.	4365 5435 1·24
9.—276 for 32 secs.	O ₂ 254 CO ₂ 216 R.Q. 0·85 F.P. 32 mins.	258 226 0·86	15447 15006 0·97 T.C. 45 mins.	3927 5061 1·29
10.—270 for 15 secs.	O ₂ 240 CO ₂ 183 R.Q. 0·76 F.P. 35 mins.	228 193 0·84	12653 12049 0·94 T.C. 45 mins.	2123 3589 1·68

since it is so unmistakable in the present experiments. The answer is presumably that in the previous experiments the exercise never reached such

severity as that involved in sprint running on a track. In recent investigations of the dynamics and the energy exchanges of sprint running Furusawa, Hill and Parkinson (2) have shown the enormous rate at which energy is expended at top speed. Fatigue rapidly sets in, a noticeable decrease of velocity being observed, even in the most highly-trained subject, after 70 yards. With exercise of such severity we might imagine some nervous or physiological excitement, of a character not usually associated with moderate exercise, resulting conceivably in an overflow of impulses to the liver and the suprarenal glands, with a resulting change in metabolism.

Table IV.—Standing-Running at High Speed. Subject, C.H.B. (Toronto).

Date.	Exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.	Oxygen requirement.
1927 Sept. 28	200 s.p.m. for 2 mins. 40 secs.	c.c. per min. O ₂ . . . 239 CO ₂ . . . 216 R.Q. . . . 0.90	c.c. per min. 248 199 0.80	c.c. 24528 26051 1.06 T.C. 56 mins.	c.c. 10920 14459 1.32	litres per min. 4.09
1928 Jan. 17	As fast as possible for 35 secs.	O ₂ . . . 243 CO ₂ . . . 187 R.Q. . . . 0.76	238 181 0.76	22282 22292 1.00 T.C. 52 mins.	9750 12724 1.30	16.70
Jan. 25	Ditto, for 30 secs.	O ₂ . . . 241 CO ₂ . . . 204 R.Q. . . . 0.84	245 197 0.80	20332 21470 1.05 T.C. 52 mins.	7696 11070 1.43	15.39
Feb. 21	Ditto, for 32 secs.	O ₂ . . . 254 CO ₂ . . . 210 R.Q. . . . 0.82	260 195 0.75	19260 20151 1.05 T.C. 50 mins. 23 secs.	6313 9975 1.58	11.80
Feb. 22	Ditto, for 30 secs.	O ₂ . . . 241 CO ₂ . . . 208 R.Q. . . . 0.86	254 192 0.75	19765 19843 1.00 T.C. 52 mins.	6921 9443 1.36	13.80

AN ANALYSIS OF THE RECOVERY PROCESS AFTER SEVERE STANDING-RUNNING.

A further analysis of the effect described could obviously be made by following the respiratory process, not by a single collection of the expired air, but by a series of collections. Seven experiments of this character have been performed on one subject, at a speed of standing-running greater than 280

steps per minute, continued until the subject was practically exhausted. The recovery period was divided into four or five parts, the time of collection in each being 10 to 15 minutes (see Table V). The total oxygen used and the total CO_2 expired during the recovery process are shown in the eighth column, while the ninth gives excess metabolism. The same high respiratory quotient of the excess metabolism is seen as before. The first collection, which includes exercise, shows invariably a high respiratory quotient, 1.23 to 1.50. In the second collection the respiratory quotient attains nearly the same level as that of the first resting reading. The subsequent collections show a slight fall of respiratory quotient, which finally rises again. This drop of respiratory quotient is less conspicuous than that described by Hill, Long and Lupton (5); in fact, in two cases 4 and 7, the respiratory quotient is above that of the first resting collection. If the large excess of CO_2 in the first interval were due to a change in the acid-base equilibrium we should expect a corresponding large fall of respiratory quotient at some time within 50 minutes, due to the subsequent retention of CO_2 initially washed out. There is, however, little evidence of any considerable retention of CO_2 or fall of respiratory quotient, the lowest value of the latter being about 0.70 in experiments 2 and 5. There is no doubt that to all intents and purposes the recovery process is complete before the beginning of the final resting reading.

Now in blood and tissues lactic acid may displace an equimolecular amount of CO_2 . If we assume that the recovery process depends solely on the oxidation of carbohydrate, and that no other metabolic process takes place, then we can calculate for this subject the extra CO_2 blown out. The quantities of the extra CO_2 are 1838, 1938, 2601, 3456, 2274, 2643 and 2495 c.c. respectively. Corresponding to this CO_2 we should expect the presence of 7.3, 7.8, 10.4, 13.8, 9.1, 10.6 and 10.0 gm. of lactic acid in the tissues at the end of an hour's recovery from exercise lasting only half a minute; these are impossible amounts, and as we have seen above, the same process can be repeated again and again, an excess of CO_2 being produced in each experiment. Thus the extra CO_2 seen in the first collection of Table V is not due to a shift in the acid-base equilibrium of blood and tissue, but to a true metabolic change induced by violent exercise.

A direct determination of the lactic acid content of the blood after exercise and recovery, completed under conditions similar to those prevailing during the above experiments, has proved the justice of our expectation that no considerable change occurs in the lactic acid between the beginning and end of a succession of experiments involving exercise and recovery. The

Table V.—Standing-Running at Extreme Speed. Division of Recovery Period into Several Intervals.
D.E. = duration of exercise in seconds. Subject, K.F. (London).

First resting value.	First collection including exercise.	Second collection.	Third collection.	Fourth collection.	Fifth collection.	Final resting value.	Total metabolism.	Excess metabolism.
c.c. per min. O ₂ ... 230 CO ₂ ... 192 R.Q. ... 0.83 F.P. ... 30 mins.	c.c. 5752 7194 1.25 T.C. 10 mins. D.E. 32 secs.	c.c. 2929 2538 0.86 10 mins.	c.c. 2650 2002 0.75 10 mins.	c.c. 2528 2135 0.71 10 mins.	c.c. 2439 1732 0.71 10 mins.	c.c. per min. 242 179 0.74	c.c. 16288 15610 0.95	c.c. 4488 6326 1.40
O ₂ ... 232 CO ₂ ... 191 R.Q. ... 0.82 F.P. ... 30 mins.	c.c. 6719 8465 1.26 T.C. 10 mins. D.E. 37 secs.	c.c. 2863 2557 0.89 10 mins.	c.c. 2743 2201 0.80 10 mins.	c.c. 2530 1802 0.71 10 mins.	c.c. 2438 1926 0.78 10 mins.	c.c. per min. 251 200 0.80	c.c. 17313 16951 0.97	c.c. 5238 7176 1.36
O ₂ ... 234 CO ₂ ... 226 R.Q. ... 0.92 F.P. ... 35 mins.	c.c. 5502 8263 1.50 T.C. 10 mins. D.E. 22 secs.	c.c. 2695 2460 0.91 10 mins.	c.c. 2408 1778 0.73 10 mins.	c.c. 2625 2229 0.88 10 mins.	c.c. 2368 1834 0.77 9½ mins.	c.c. per min. 246 202 0.82	c.c. 15498 16564 1.06	c.c. 3618 6219 1.71
O ₂ ... 224 CO ₂ ... 160 R.Q. ... 0.71 F.P. ... 60 mins.	c.c. 7556 9848 1.30 T.C. 15 mins. D.E. 22 secs.	c.c. 3719 2900 0.78 14 mins.	c.c. 3843 2959 0.77 15 mins.	c.c. 3620 2982 0.82 15 mins.	c.c. 2313 1799 0.77 10½ mins.	c.c. per min. 245 195 0.79	c.c. 21051 20488 0.97	c.c. 4519 7975 1.76
O ₂ ... 237 CO ₂ ... 206 R.Q. ... 0.87 F.P. ... 35 mins.	c.c. 7686 9798 1.27 T.C. 15 mins. D.E. 30 secs.	c.c. 2815 2317 0.82 10 mins.	c.c. 2794 2167 0.77 10 mins.	c.c. 2784 2026 0.72 10 mins.	c.c. 2514 1734 0.69 10 mins.	c.c. per min. 257 186 0.72	c.c. 18573 18042 0.97	c.c. 4988 7262 1.45
O ₂ ... 273 CO ₂ ... 238 R.Q. ... 0.87 F.P. ... 35 mins.	c.c. 8335 11242 1.34 T.C. 15½ mins. D.E. 34 secs.	c.c. 4403 3590 0.81 14½ mins.	c.c. 4219 3175 0.75 15 mins.	c.c. 4193 3446 0.82 15 mins.	c.c. 2514 1734 0.69 10 mins.	c.c. per min. 272 229 0.84	c.c. 21150 21453 1.01	c.c. 4800 7443 1.55
O ₂ ... 287 CO ₂ ... 214 R.Q. ... 0.74 F.P. ... 40 mins.	c.c. 8890 10932 1.23 T.C. 15 mins. D.E. 28 secs.	c.c. 4657 3500 0.75 15 mins.	c.c. 4634 3428 0.74 16 mins.	c.c. 4150 3315 0.80 14 mins.	c.c. 2514 1734 0.69 10 mins.	c.c. per min. 289 240 0.83	c.c. 22340 21175 0.94	c.c. 5060 7555 1.49

subjects, the duration of exercise and the intervals of recovery were as follows :—

Subject :	K.F.	...	J.L.P.
D.E.	37 secs.		39 secs.
Interval		30 mins.	
D.E.	61 secs.		46 secs.
Interval		30 mins.	
D.E.	80 secs.		64 secs.
Interval		45 mins.	
D.E.	48 secs.		45 secs.
Interval		30 mins.	
D.E.	83 secs.		54 secs.
Interval		60 mins.	

Samples of blood were drawn before the exercise and after the final rest. The quantity of lactic acid found (milligrams per 100 cubic centimetres) were :—

	K.F.	J.L.P.
Initial	23	15
Final	19	21

As expected, there was no sign of any important increase in the lactic acid content of the blood.

THE RESPIRATORY QUOTIENT OF THE EXCESS METABOLISM DUE TO MODERATE EXERCISE.

In order to test whether the high excess respiratory quotients obtained at Ithaca were due to climatic conditions (for example to iodine deficiency) affecting the subjects under the conditions prevailing at the place, the following experiments were undertaken there (see Table VI). The exercise was slow running, usually in the corridor of the Baker Laboratory, carrying a 100-litre bag in the hand. Without any further explanation this table shows that the respiratory quotient of the excess metabolism due to exercise of this character and of short duration is about unity, thus confirming the results (6) (1) obtained in 1924 and 1925.

Similar observations were made later in London to confirm this point. The exercise adopted was standing-running, and the results at low speed are given in Table VII. The six observations there recorded were inserted at

Table VI.—Slow Running. (Ithaca.)

Subject, exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.	Excess R.Q.
K.F., 400 yds. in 2 mins. 16 secs. Track.	c.c. per min. O ₂ 250 CO ₂ 199 R.Q. 0.80	c.c. per min. 240 198 0.83	c.c. 17475 15245 0.87 T.C. 50 mins.	c.c. 5225 5290	1.01
J.L.P., 400 yds. in 1 min. 52 secs. Track.	O ₂ 280 CO ₂ 227 R.Q. 0.81	276 216 0.78	19243 16500 0.86 T.C. 50 mins.	5343 5425	1.02
K.F., 300 yds. in 1 min. 55 secs. Corridor.	O ₂ 193 CO ₂ 154 R.Q. 0.80	202 167 0.82	14680 12907 0.87 T.C. 50 mins.	4805 4882	1.01
J.L.P., 500 yds. in 4 mins. 15 secs. Corri- dor.	O ₂ 275 CO ₂ 231 R.Q. 0.84	270 206 0.78	16142 13714 0.85 T.C. 40 mins.	5242 4974	0.95
J.L.P., 300 yds. in 1 min. 40 secs. Corridor.	O ₂ 275 CO ₂ 210 R.Q. 0.76	275 209 0.76	18143 15207 0.84 T.C. 50 mins.	4393 4732	1.07
K.F., 300 yds. in 1 min. 30 secs. Corridor.	O ₂ 216 CO ₂ 159 R.Q. 0.74	211 165 0.78	15246 12738 0.84 T.C. 50 mins.	4571 4638	1.01
J.L.P., 300 yds. in 1 min. 40 secs.	O ₂ 271 CO ₂ 220 R.Q. 0.81	277 219 0.79	18483 15825 0.86 T.C. 50 mins.	4783 4850	1.01

random between other experiments which have been described in the section dealing with the extremely high speed exercise.

The last column shows that for exercise of this character the respiratory quotient of the excess metabolism is approximately unity, thus confirming the previous observations. The result, moreover, proves that in this respect the bodily condition of one at any rate of our subjects was essentially the same (*a*) in 1924 and 1925 in London, (*b*) in Ithaca, and (*c*) in London in 1927.

While the last-mentioned experiments were being performed, similar observations were being made in Toronto by two of us (Best and Ridout). In these cases standing-running at a speed of 132 steps per minute (with subject, C.H.B.) as well as pedalling a bicycle ergometer with a friction of 900 gms. (subject,

Table VII.—Standing-Running at Low Speed. Subject, K.F. (London).

Exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.
steps per min.	c.c. per min.	c.c. per min.	c.c.	c.c.
1.—148 for 3 mins.	O ₂ 235 CO ₂ 224 R.Q. 0·93 F.P. 30 mins.	236 222 0·94	13249 13095 0·98 T.C. 34 mins.	5242 5513 1·04
2.—186 for 3 mins.	O ₂ 219 CO ₂ 198 R.Q. 0·89 F.P. 33 mins.	237 198 0·83	17539 17496 1·00 T.C. 41 mins.	8191 9378 1·14
3.—136 for 5 mins.	O ₂ 210 CO ₂ 191 R.Q. 0·91 F.P. 40 mins.	213 203 0·95	19441 18575 0·95 T.C. 60 mins.	6751 6755 1·00
4.—136 for 3 mins.	O ₂ 216 CO ₂ 193 R.Q. 0·88 F.P. 39 mins.	220 202 0·92	10019 9503 0·94 T.C. 30 mins.	3479 3578 1·02
5.—143 for 3 mins.	O ₂ 247 CO ₂ 202 R.Q. 0·81 F.P. 35 mins.	245 217 0·88	10736 9561 0·89 T.C. 33 mins.	2618 2648 1·01
6.—134 for 5 mins.	O ₂ 201 CO ₂ 168 R.Q. 0·83 F.P. 20 mins.	202 167 0·82	15469 13916 0·89 T.C. 35 mins.	8417 8054 0·96

E.McH.) were studied. See Table VIII. In the former case the duration of exercise varied from 1 to 8 minutes, and the oxygen requirement was between 2 and 2·8 litres per minute. In the latter case 4 and 8 minutes pedalling were performed, with an oxygen requirement of somewhere about 0·7 litres per minute. The respiratory quotient of the excess metabolism, in all the experiments except two, was approximately unity.

Table VIII.—Standing-Running at Moderate Speed (Subject, C.H.B.), and Pedalling of a Bicycle Ergometer at Moderate Speed (Subject, E.McH.).

Date.	Subject, exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.	Oxygen requirement.
1927 July 6	C.H.B., s.r., 132 for 2 mins.	c.c. per min. O ₂ ... 238 CO ₂ ... 204 R.Q. ... 0.85	c.c. per min. 251 199 0.79	c.c. 13124 12980 0.99 T.C. 30½ mins.	c.c. 5652 6850 1.21	litres per min. 2.82
Sept. 13	Ditto, ditto, for 1 min.	O ₂ ... 243 CO ₂ ... 185 R.Q. ... 0.76	256 197 0.77	10890 9197 0.84 T.C. 33 mins.	2673 2894 1.07	2.67
Sept. 8	Ditto, ditto, for 3 mins.	O ₂ ... 195 CO ₂ ... 159 R.Q. ... 0.81	220 178 0.81	13913 12600 0.91 T.C. 32½ mins.	7153 7150 1.00	2.38
Sept. 7	Ditto, ditto, for 5 mins.	O ₂ ... 257 CO ₂ ... 213 R.Q. ... 0.83	233 187 0.80	19888 17808 0.89 T.C. 40 mins.	10088 9808 0.97	2.01
Sept. 14	Ditto, ditto, for 8 mins.	O ₂ ... 246 CO ₂ ... 202 R.Q. ... 0.82	257 194 0.76	29219 26799 0.92 T.C. 46 mins.	17673 17691 1.00	2.20
Sept. 15	E.McH., b.c. for 4 mins.	O ₂ ... 220 CO ₂ ... 188 R.Q. ... 0.85	226 187 0.83	12032 10566 0.88 T.C. 40½ mins.	3001 2952 0.98	0.75
Sept. 16	Ditto, ditto, for 4 mins.	O ₂ ... 217 CO ₂ ... 173 R.Q. ... 0.80	211 170 0.80	11848 10076 0.84 T.C. 42 mins.	2860 2894 1.01	0.71
Oct. 31	Ditto, ditto, for 4 mins.	O ₂ ... 213 CO ₂ ... 187 R.Q. ... 0.88	214 182 0.85	12612 11397 0.90 T.C. 45½ mins.	2843 2980 1.01	0.71
Dec. 16	Ditto, ditto, for 8 mins.	O ₂ ... 205 CO ₂ ... 169 R.Q. ... 0.82	206 176 0.85	15417 12672 0.82 T.C. 45 mins.	6147 4932 0.80	0.77
Dec. 21	Ditto, ditto, for 8 mins.	O ₂ ... 176 CO ₂ ... 149 R.Q. ... 0.85	187 162 0.86	14433 13190 0.91 T.C. 48 mins.	5745 5750 1.00	0.71

THE LOW EXCESS RESPIRATORY QUOTIENT DUE TO VERY MODERATE
EXERCISE.

During the period from June to December, 1927, Best and Ridout in Toronto made experiments in order to test whether the statement made by Furusawa in 1925 can be applied to very moderate exercise. Altogether 29 observations were recorded on two subjects, with standing-running and on a bicycle ergometer. The results are tabulated below :—

Table IX. —Standing-Running at Low Speed, Stepping Low. Subject, C.H.B. (Toronto).

Date.	Exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.	Oxygen requirement.
1927	steps per min.	c.c. per min.	c.c. per min.	c.c.	c.c.	litres per min.
July 8	132, for 5 mins.	O ₂ 239 CO ₂ 193 R.Q. 0.81	242 197 0.81	13746 11580 0.84 T.C. 30 mins.	6525 5733 0.88	1.30
July 21	Ditto, for 6 mins.	O ₂ 234 CO ₂ 186 R.Q. 0.79	234 187 0.80	13987 11245 0.80 T.C. 32 mins.	6490 5274 0.81	1.08
July 12	Ditto, for 8 mins.	O ₂ 234 CO ₂ 196 R.Q. 0.83	223 186 0.83	21279 18579 0.87 T.C. 46 mins.	10768 9793 0.90	1.34
July 14	Ditto, for 12 mins.	O ₂ 238 CO ₂ 195 R.Q. 0.82	229 200 0.87	27635 22825 0.82 T.C. 50 mins.	15935 12935 0.80	1.33
July 18	Ditto, for 12 mins.	O ₂ 207 CO ₂ 179 R.Q. 0.86	212 177 0.83	23682 20423 0.86 T.C. 48½ mins.	13512 11775 0.87	1.12
July 13	Ditto, for 15 mins.	O ₂ 243 CO ₂ 194 R.Q. 0.80	233 175 0.76	33155 28249 0.85 T.C. 55½ mins.	20026 18044 0.90	1.33
July 19	Ditto, for 20 mins.	O ₂ 235 CO ₂ 181 R.Q. 0.77	228 183 0.80	36429 29322 0.80 T.C. 61 mins.	22307 18214 0.82	1.11
Dec. 6	Ditto, for 3 mins.	O ₂ 242 CO ₂ 200 R.Q. 0.82	240 194 0.81	12544 10395 T.C. 35 mins.	4109 3503 0.85	1.33

Table X.—Bicycle Ergometer. Subjects, C.H.B. (the first four experiments) and E.McH. (Toronto).

Date.	Exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.	Oxygen requirement.
1927	rev. per min.	c.c. per min.	c.c. per min.	c.c.	c.c.	litres per min.
June 2	127 for 2 mins. . .	O ₂ . . . 249 CO ₂ . . . 215 R.Q. . . . 0.86	247 206 0.84	11975 10160 0.80 T.C. 33 mins.	3801 3210 0.84	1.90
June 3	134 for 1 min.	O ₂ . . . 218 CO ₂ . . . 186 R.Q. . . . 0.85	221 179 0.81	8358 7003 0.84 T.C. 31 mins.	1556 1355 0.87	1.56
June 15	191 for 4 mins. . .	O ₂ . . . 249 CO ₂ . . . 196 R.Q. . . . 0.78	238 201 0.84	14168 12115 0.85 T.C. 40½ mins.	4351 4126 0.94	1.09
June 27	181 for 3 mins. . .	O ₂ . . . 252 CO ₂ . . . 200 R.Q. . . . 0.80	248 190 0.76	10949 9036 0.82 T.C. 31 mins.	3205 3000 0.93	1.07
Sept. 22	65 for 4 mins. . .	O ₂ . . . 220 CO ₂ . . . 175 R.Q. . . . 0.79	228 184 0.81	10368 8368 0.81 T.C. 38½ mins.	1785 1482 0.83	0.44
Oct. 24	68 for 4 mins. . .	O ₂ . . . 220 CO ₂ . . . 190 R.Q. . . . 0.86	218 193 0.88	11054 9672 0.87 T.C. 42 mins.	1865 1638 0.87	0.44
Oct. 10	69 for 4 mins. . .	O ₂ . . . 227 CO ₂ . . . 183 R.Q. . . . 0.80	233 186 0.80	11881 9731 0.82 T.C. 43 mins.	1990 1797 0.90	0.49
Oct. 18	71 for 4 mins. . .	O ₂ . . . 207 CO ₂ . . . 176 R.Q. . . . 0.85	213 184 0.86	10452 8844 0.84 T.C. 40 mins.	2064 1640 0.80	0.52
Sept. 23	76 for 4 mins. . .	O ₂ . . . 225 CO ₂ . . . 192 R.Q. . . . 0.86	237 191 0.80	9542 7959 0.83 T.C. 32½ mins.	2041 1729 0.85	0.51
Sept. 27	84 for 4 mins.	O ₂ . . . 228 CO ₂ . . . 192 R.Q. . . . 0.84	210 171 0.83	11095 9147 0.82 T.C. 41 mins.	2119 1701 0.80	0.53
Nov. 1	105 for 4 mins. . .	O ₂ . . . 211 CO ₂ . . . 176 ? R.Q. . . . 0.83	215 184 0.85	12514 10890 0.87 T.C. 45 mins.	2925 2781 0.96	0.73

Table X—continued.

Date.	Exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.	Oxygen requirement.
1927	rev. per min.	c.c. per min.	c.c. per min.	c.c.	c.c.	litres per min.
Nov. 9	102 for 4 mins.	O ₂ . . . 227 CO ₂ . . . 194 R.Q. . . . 0.85	225 197 0.87	12320 10859 0.88 T.C. 44 mins.	2370 2266 0.95	0.59
Nov. 4	104 for 4 mins.	O ₂ . . . 205 CO ₂ . . . 178 R.Q. . . . 0.85	206 184 0.88	12969 11398 T.C. 52 mins.	2308 1971 0.85	0.57
Nov. 3	106 for 4 mins.	O ₂ . . . 211 CO ₂ . . . 184 R.Q. . . . 0.87	212 179 0.85	12528 10790 0.86 T.C. 48 mins.	2376 2073 0.88	0.59
Sept. 29	106 for 4 mins.	O ₂ . . . 234 CO ₂ . . . 188 R.Q. . . . 0.80	229 191 0.83	11864 9956 0.84 T.C. 40 mins.	2020 2396 0.91	0.65
Oct. 5	111 for 4 mins.	O ₂ . . . 229 CO ₂ . . . 191 R.Q. . . . 0.83	246 197 0.80	12414 10489 0.85 T.C. 42½ mins.	2316 2227 0.95	0.58
Nov. 10	65 for 11 mins.	O ₂ . . . 219 CO ₂ . . . 179 R.Q. . . . 0.83	236 188 0.80	16181 13714 0.85 T.C. 51½ mins.	4470 4238 0.94	0.41
Nov. 11	60 for 11 mins.	O ₂ . . . 214 CO ₂ . . . 180 R.Q. . . . 0.83	216 183 0.85	15723 13477 0.85 T.C. 51½ mins.	4640 4151 0.89	0.42
Nov. 14	65 for 11 mins.	O ₂ . . . 216 CO ₂ . . . 195 R.Q. . . . 0.90	215 190 0.88	15605 13821 0.88 T.C. 52 mins.	4388 3811 0.87	0.40
Nov. 15	69 for 11 mins.	O ₂ . . . 222 CO ₂ . . . 182 R.Q. . . . 0.82	227 190 0.83	16238 13939 0.86 T.C. 52½ mins.	4467 4173 0.93	0.40
Nov. 17	67 for 11 mins.	O ₂ . . . 228 CO ₂ . . . 184 R.Q. . . . 0.80	210 181 0.86	15938 12994 0.81 T.C. 52 mins.	4529 3489 0.77	0.41

In Table IX the exercise consisted of standing-running at a constant speed of 132 steps per minute, stepping low, and maintained for 3 to 20 minutes. It was consistently found that the excess respiratory quotient is lower than unity,

as shown in the sixth column. In fact some values are nearly equal to the basal respiratory quotient. The mean respiratory quotient of the basal metabolism is 0.81 and that of the excess metabolism is just higher than the former, being 0.85. This result was confirmed with the same subject pedalling a bicycle ergometer (friction 900 gms., 127 to 191 revolutions per minute). The subject, E.McH., pedalling the bicycle ergometer, showed the same effect at low speed. A very slow rate of 65 to 111 revolutions per minute was adopted, in view of the fact that this subject already showed a high respiratory quotient at a lower speed than that employed by the subject C.H.B. (see Table X). The excess respiratory quotient attains a slightly higher level than the basal metabolism, except in four cases where it is slightly lower. The mean values for all the observations are 0.87 and 0.84 respectively.

These results are directly contradictory to the data just described and to the observations reported by Furusawa in the previous paper, where it was suggested that carbohydrate alone is responsible for the energy supply of muscular movement of short duration, and that fat or other food-stuffs can be used only after conversion into carbohydrate. To reconcile the two sets of observations it might be suggested that the excess metabolism is only such a small fraction of the total metabolism in the case of this very mild exercise, that small errors in the estimation of the quantities involved might lead to erroneous results. The large number of observations, however, and their consistency among themselves would seem to weaken this objection. We have already pointed out above the important rôle played by the subject in such experiments as these. This is especially true in the case of very mild exercise, such as we have just described. A small change in the respiratory movements, or in the muscle tone, might be a source of serious disturbance in the excess metabolism. The consistent results obtained, however, in such a large number of observations, could hardly be attributed to conscious or unconscious adjustment of respiratory movements, or of muscle tone, on the part of the subjects.

There is an agreement between the two subjects used in the series of experiments of Table X that the respiratory quotient of the excess metabolism is invariably lower than unity; there is, however, a difference clearly seen between them in respect of the exact level of severity of exercise at which the respiratory quotient of the excess metabolism rises to unity. In the case of C.H.B., an oxygen requirement of 1.0 to 1.9 litres per minute shows the lower level of the excess respiratory quotient, whereas E.McH. exhibits a value near unity at an oxygen requirement of the order of 0.7 litre per minute. We must

assume, therefore, that the severity of exercise showing the low level of excess respiratory quotient varies slightly according to the individual.

A question now arises whether the results obtained by Furusawa in 1925 were due to the possibility that the exercise employed was not moderate enough to show this effect with the particular subject employed. Accordingly 11 experiments were performed in London with this subject (K.F.), the results of which are given in Table XI. The respiratory quotient of the excess

Table XI.—Excess Metabolism of Very Mild Exercise. Subject, K.F. (1928 in London).

Exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.
	c.c. per min.	c.c. per min.	c.c.	c.c.
steps per min. 108 (walking), 10 mins.	O ₂ 240 CO ₂ 191 R.Q. 0.79 T.C. 10 mins. F.P. 50 mins.	248 190 0.76 10 mins.	15115 12041 0.79 45 mins.	4135 3469 0.83
98 (walking), 9 mins.	O ₂ 228 CO ₂ 180 R.Q. 0.78 T.C. 10 mins. F.P. 50 mins.	240 187 0.78 10 mins.	17378 13714 0.79 50 mins.	5678 4539 0.82
106, 10 mins. (15 mins. pre- vious exercise).	O ₂ 204 CO ₂ 153 R.Q. 0.75 T.C. 10 mins. F.P. 35 mins.	205 154 0.75 9 mins.	14359 12248 0.85 40 mins.	6179 6108 0.99
106, 10 mins. (15 mins. pre- vious exercise)	O ₂ 241 CO ₂ 195 R.Q. 0.81 T.C. 10 mins. F.P. 30 mins.	246 194 0.78 10 mins.	15421 13720 0.88 40 mins.	5681 5940 1.04
106, 10 mins. (15 mins. pre- vious exercise).	O ₂ 258 CO ₂ 186 R.Q. 0.72 T.C. 12 mins. F.P. 30 mins.	257 185 0.72 11 mins.	16065 12927 0.80 45 mins.	4478 4580 1.02
106, 10 mins. (15 mins. pre- vious exercise).	O ₂ 243 CO ₂ 189 R.Q. 0.77 T.C. 11 mins. F.P. 30 mins.	243 188 0.77 10 mins.	13683 11239 0.82 40 mins.	3968 3699 0.93
84, 1 min.	O ₂ 249 CO ₂ 206 R.Q. 0.82 T.C. 10 mins. F.P. 47 mins.	230 188 0.81 10 mins.	4259 3454 0.81 15 mins.	667 499 0.74

Table XI—(continued).

Exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.
steps per min.	c.c. per min.	c.c. per min.	c.c.	c.c.
104, 10 mins. (15 mins. previous exercise).	O ₂ . . . 226 CO ₂ . . . 171 R.Q. . . . 0.75 T.C. . . . 10 mins. F.P. . . . 40 mins.	226 178 0.87 11 mins.	13941 11336 0.81 42 mins.	4449 4007 0.90
106, 30 mins.	O ₂ . . . 230 CO ₂ . . . 172 R.Q. . . . 0.75 T.C. . . . 20 mins. F.P. . . . 33 mins.	225 170 0.75 20 mins.	27981 23159 0.82 78 mins.	10236 9821 0.95
106, 20 mins.	O ₂ . . . 220 CO ₂ . . . 162 R.Q. . . . 0.73 T.C. . . . 21 mins. F.P. . . . 45 mins.	210 162 0.74 10 mins.	18869 15224 0.80 50 mins.	7894 7124 0.90
106, 30 mins.	O ₂ . . . 249 CO ₂ . . . 201 R.Q. . . . 0.80 T.C. . . . 15 mins. F.P. . . . 30 mins.	223 177 0.79 10 mins.	24018 20025 0.84 60 mins.	9858 8685 0.88

metabolism shows somewhat wide variation, three observations being near or just above unity and the others scattered below it. It is easily seen at any rate that the respiratory quotient of the excess metabolism is not invariably unity; it often shows a low value approaching that of the resting metabolism. Where, however, the oxygen requirement of exercise is so small (less than 0.5 litre per minute) a small error in the resting metabolism (say 2 or 3 c.c. per minute) might provide a large error in the respiratory quotient of the excess metabolism, especially when the recovery period allowed is long, and it was possible that a 10-minutes sample for the determination of the resting metabolism might not be sufficient for this special type of experiments. To test this possibility, six experiments were performed (see Table XII). The duration of the first resting collection was 40 to 60 minutes, so as to eliminate possible errors due to irregularity of breathing, etc. The resting values are given in cubic centimetres per hour. The first three observations were made at 106 steps per minute, the others were at a somewhat higher speed of 130 steps per minute. The perfect accordance obtained in a given set of resting respiratory quotients is remarkable. The excess respiratory quotient has a very low value for the

Table XII.—Excess Metabolism of Very Mild Exercise with Long Period of Resting Collection. Subject, K.F. (1928, London).

Exercise.	First resting value.		Second resting value.	Total metabolism.	Excess metabolism.
steps per min.	c.c. per hour.		c.c. per hour.	c.c.	c.c.
106, 30 mins. . .	O ₂	13230	12796	22323	9310
	CO ₂	11070	10612	18176	7335
	R.Q.	0·83	0·83	0·81	0·78
	T.C.	60 mins.	60 mins.	60 mins.	
	F.P.	30 mins.			
106, 29 mins. . .	O ₂	12003	13024	21114	8601
	CO ₂	9250	10050	16109	6459
	R.Q.	0·77	0·77	0·76	0·75
	T.C.	55 mins.	55 mins.	60 mins.	
	F.P.	30 mins.			
106, 30 mins.	O ₂	14105	13834	29078	9290
	CO ₂	11521	11180	22240	6167
	R.Q.	0·81	0·81	0·76	0·66
	T.C.	55 mins.	35 mins.	85 mins.	
	F.P.	40 mins.			
130, 10 mins.	O ₂	14385	14423	24101	5617
	CO ₂	11011	10920	19815	5744
	R.Q.	0·76	0·75	0·82	1·02
	T.C.	60 mins.	40 mins.	77 mins.	
	F.P.	55 mins.			
130, 13 mins.	O ₂	13450	13490	21600	8130
	CO ₂	11308	11048	18596	7418
	R.Q.	0·84	0·82	0·86	0·91
	T.C.	60 mins.	40 mins.	60 mins.	
	F.P.	40 mins.			
130, 5 mins.	O ₂	15761	14944	17879	2527
	CO ₂	12164	11616	14620	2730
	R.Q.	0·77	0·77	0·81	1·08
	T.C.	60 mins.	60 mins.	60 mins.	
	F.P.	60 mins.			

low speed, while that of the moderate speed is higher. Thus we come again to the conclusion that the respiratory quotient of the excess metabolism resulting from very mild exercise is lower than unity, while that of moderate exercise tends to approximate to unity.

DISCUSSION.

With the discovery of the new and unexpected phenomenon connected with the excess respiratory quotient of extremely violent exercise, followed by the results obtained in very mild exercise, our conception of the gaseous metabolism of exercise must be largely modified. As pointed out at the

beginning of the present paper, the respiratory quotient at a given instant is not the combustion coefficient of foodstuffs. It cannot be regarded as such unless very special conditions be observed. This view, already put forward in the papers by Hill, Long and Lupton (1924) and by Furusawa (1925), has recently been emphasised by Cathcart and Markowitz (3), and by Bock, Dill, Fölling, Van Caulaert and Hurxthal (7). For very mild exercise the excess respiratory quotient starts at the same level as the resting value, gradually rises to unity at moderate exercise, then attains a very high value (up to 1.70) at the most severe exercise. It has been shown that the high respiratory quotient of the excess metabolism is not due to the "blowing off" of CO_2 by over-ventilation or by lactic acid. With a subject of 50 kilograms body-weight it is possible to blow off 2.5 litres of CO_2 by extreme forced breathing. Actual figures obtained are: 2.7, 1.8, 2.2, 1.8, 2.0 and 2.7 litres, with a period of forced breathing varying from 1.2 to 15 minutes. We have failed, however, to observe any sign of dyspnoea after recovery from exercise in any of our subjects, undertaking the most severe exercise. The lactic acid determinations also failed to show any accumulation of acid in the blood after recovery from a series of such exercises performed at appropriate intervals. The absence of retention of CO_2 during the recovery process, and the fact that the phenomenon can be observed in a succession of experiments, give strong support to the view that the excess CO_2 is not simply preformed CO_2 driven off. We must conclude, therefore, that the high excess respiratory quotient is really caused by some metabolic change induced by very violent exercise.

In moderate exercise the respiratory quotient of the excess metabolism is round about unity and thus agrees with the report made by one of us in 1925. When exercise is very mild this respiratory quotient shows lower values than unity and finally reaches the same level as that of the resting metabolism. These results have recently been confirmed quite independently by Bock, Dill, Fölling, Van Caulaert and Hurxthal (7) during their studies of muscular exercise. They investigated the "steady state" of exercise, in the sense proposed by Hill and Lupton (8), paying attention to the metabolic processes occurring inside the body in a new "environment," that of exercise. Following is a quotation from their paper: "Similarly, during continuous muscular exercise 'a steady state' is reached when the demand of oxygen is adequately met. Such a steady state implies a relatively constant total ventilation elimination only of carbon dioxide produced in metabolism, steady pulse and respiratory rate and a constant internal environment. These conditions may be associated with an oxygen debt, provided it is not cumulative but is chiefly

acquired in the period from the beginning of work until the steady state is reached." With this definition of a steady state of exercise, which is essentially the same as that of Hill and Lupton, we may use the respiratory quotient of the gaseous metabolism accompanying exercise as an indicator of the metabolic changes occurring inside the body during exercise. In Table VI of their fourth paper, they have shown clearly that the respiratory quotient of a steady state of exercise is a function of the oxygen requirement, increasing gradually from the level of the resting value up to unity at an oxygen intake of about 2 litres per minute. In the case of Demar, a "Marathon" runner in constant training, this rise of respiratory quotient with increasing oxygen requirement was less conspicuous than in the other three subjects and never reached unity. It is clear that whether we take, as an indicator of the metabolic changes accompanying exercise, the respiratory quotient of the excess metabolism on the one hand, or the respiratory quotient of the steady state on the other, we come to the same conclusion, namely, that as the intensity of exercise increases the respiratory quotient rises from the resting level to unity or near it, provided that exercise remains within such a limit of severity that a steady state can be attained. Thence, as shown in the earlier part of this paper, it continues to rise over unity to a remarkably high value. The result obtained by Furusawa in 1925 is, therefore, only a special case of the general metabolic changes accompanying exercise.

In recent years, several papers have appeared dealing with the present question. At the general meeting of the American Physiological Society in Rochester, N.Y. (1927), Marsh (9) reported results which appeared to show a large divergence in different experiments between the several values of the respiratory quotient of the excess metabolism, which, if correct, would indicate that all the foodstuffs can be used directly for the recovery process following muscular contraction. The lack, however, of agreement in her results suggests a doubt whether all the precautions discussed in our introductory remarks were observed. Reynold, Sevringhaus and Stark (10) have repeated the experiments of Krogh and Lindhard (11) and found no difference in the efficiency on different foodstuffs. They did not measure their respiratory quotients, so it is impossible to see how far the respiratory quotient of the resting metabolism had fallen during the fat diet. In a recent paper Lindhard (12) raised objections to a preliminary discussion of the present subject by Furusawa, Hill, Long and Lupton (6), and stated that their working hypothesis would not hold. From the results in Table II of his paper, it is difficult to see whether the precautions necessary for this kind of experiments were carefully observed.

His objection that the severity of exercise was measured by oxygen requirement, and not in mechanical units, need scarcely be answered ; it would be a pity if physiologists were forbidden to investigate any form of exercise other than that employing the bicycle ergometer. On the other hand, Simonson (13), using a Zuntz-Geppert's apparatus, reported a high respiratory quotient of the excess metabolism as the result of very mild muscular efforts. The amount of oxygen involved in his experiments was so small that it is not easy to assess the value of his results. Wilson, Levine, Rivkin and Berliner (14) have studied the excess metabolism of children. In normal children the respiratory quotient of the excess metabolism due to the exercise was only slightly higher than the basal value. Rapport and Ralli (15) have recently reported the results of experiments in which the excess metabolism of mild exercise in dogs was investigated. The animal ran on a treadmill inside a calorimeter : the results of these studies show that the quotient of the excess metabolism of very mild exercise in dogs is much below unity. They did not study the excess metabolism in more severe exercise and therefore missed the higher quotients.

Many of the experimental results obtained on isolated muscle, whether by chemical or by myothermic methods, have been in favour of the view that carbohydrate alone is oxidised in the recovery process from muscular contraction. On the other hand, observations on the animal as a whole, especially in the case of the human subject, do not often exhibit the simplicity of the process involved in muscular activity. The present results show clearly, in the case of mild or very mild exercise, that carbohydrate is *not* the sole foodstuff involved in muscular exercise. Does this mean that isolated muscle is incapable of using fat directly for this purpose, and yet muscle inside the body can utilise fat directly as well as carbohydrate ? The well-planned and beautifully-made experiments of Krogh and Lindhard (11) indicate a negative answer to this question. Fat is 11 per cent. less efficient than carbohydrate as the energy source of muscular exercise. This suggests naturally a conversion of fat into carbohydrate before it is used for the purpose. One of us (K.F.) has shown that even on a fatty diet where the resting respiratory quotient attains the level of combustion of fat, the excess respiratory quotient resulting from moderate exercise of short duration is unity. Moreover, Hetzel and Long (16) found in diabetic persons that the respiratory quotient of the excess metabolism may attain a value near unity, provided that an ample supply of insulin is given.

In the paper cited above (7), Bock and others have found that respiratory quotients approximating to unity can be maintained for considerable periods

(30 to 70 minutes) during a steady state of exercise. If we were to assume that the respiratory quotient of the basal metabolism is 0.85 and that all the foodstuffs present inside the body of their subjects could be used directly to provide muscular energy; if, in other words, the combustion coefficient of the foodstuffs used for exercise were exactly the same as the respiratory quotient of the body at rest; then we should be driven to the conclusion that the high respiratory quotients observed by them must be attributed to a "blowing off" of CO_2 during exercise. We can calculate the amount of the CO_2 thus supposed to be blown off during a steady state of exercise.

The following figures represent a rough estimate of this quantity in the experiments they quote [(7) part IV]; from Table II, 6.2 Ls. (or 13.2 Ls. if we take the basal R.Q., 0.75 given in the table instead of the assumed 0.85); from Table III, 13.7, 3.3, 11.4, 10.2, 6.5; from Table IV, 3.2, 2.6, 7.4, 4.0, 6.6 Ls. Now it is inconceivable that a subject weighing 70 kilograms or so should be capable of blowing off more than 3 litres of CO_2 without any sign of dyspnoea. Moreover, there is a small oxygen debt at the end of exercise, which is to be attributed to the lag in oxygen intake before the steady state is obtained. We can, therefore, draw only one conclusion from these observations by Boek and his colleagues, the one they draw themselves, viz., that their respiratory quotients indicate a genuine effect occurring in the body metabolism during a steady state of exercise. Their evidence is strongly in favour of the hypothesis that carbohydrate is the primary fuel required to provide the energy for muscular exercise.

The respiratory quotient of the excess metabolism accompanying exercise, as we have shown, does not invariably attain the value of unity; it is a function of the oxygen requirement, rising gradually from the level of the basal metabolism to a very high value. It seems impossible to explain this gradually changing nature of the excess metabolism, especially the high values observed, by any simple assumptions such as combustion of carbohydrate and washing out of CO_2 , or by the hypothesis that the body can utilise any foodstuffs available in order to provide energy for muscular contraction. In 1911 Cannon, Sohl, and Wright (17) showed that there is a kind of glycosuria caused by emotional excitement in the cat, and later Bulatao and Cannon (18) demonstrated that the suprarenal glands are responsible for the emotional hyperglycemia. From these results we may infer that under emotional excitement sugar is mobilised in the blood and that the suprarenal glands are responsible for this mobilization. If a comparatively large quantity of sugar were made available and—not being required, owing to the sudden termination of the

exercise—were replaced partly as glycogen, but largely in the form of some substance poor in oxygen such as fatty acid, the results we have obtained could be explained. For example in Experiment 14, Table II, there is approximately 10 litres of CO_2 which cannot be accounted for by a shift of the acid-base equilibrium of the body. This experiment consisted of three 150 yards runs. If, as a result of each run, 8 grams of sugar were transformed into fat the 10 litres of CO_2 would be set free. In such experiments as we have described, made under laboratory conditions though sometimes on the track, there exists no obvious emotional excitement, though the exercise was of extreme severity.

A direct investigation of this supposed mobilization of sugar into the blood stream gave negative results, as shown in Table XIII. The exercise in these experiments was standing-running at extreme speed. A third experiment in which the subject ran 100 yards at top speed on the track gave similar results. The excess metabolism was determined in the two standing-running experiments and the quotients show high values, as before, yet there is hardly any important change observable in the blood sugar content. If 8 grams of sugar were suddenly liberated into the blood stream of a man weighing 80 kilograms (subject, C.H.B.) there is no doubt that the increase in sugar content would be easily detected, but if this amount was made available slowly during the first part of the recovery process as well as during the exercise, its effect might be overshadowed by other processes which demand sugar from the blood. Since, however, we obtained no evidence of a liberation of sugar in our experiments we do not feel justified, at present, in attempting to explain the high respiratory quotients by a mobilization of sugar and a subsequent conversion of a part of this into fat.

In calculating the respiratory quotient of the excess metabolism of muscular exercise, it is assumed that the metabolism of tissues other than the active muscles remains constant during the period of the exercise and recovery from it. We have no way of knowing whether or not the metabolism of tissues other than the active muscles does remain constant. If we disregard, for the moment, the quotient of the *excess* metabolism and calculate the quotient of the *total* metabolism of exercise and recovery, it is found that in the experiments in which very severe exercise was studied a value of approximately unity was obtained. Obviously, the exact moment of completed recovery must be determined as accurately as possible when the total metabolism is studied, since inclusion of the after-basal period would lower the respiratory quotient of the total. A second point which requires emphasis is that although we speak of the respiratory quotient of the total metabolism having a value of unity we

Table XIII.—Blood Sugar in the Recovery Period from Severe Standing-Running. Subject, C.H.B. (Toronto).

Date.	Exercise.	First resting value.	Second resting value.	Total metabolism.	Excess metabolism.	Blood sugar.
Jan. 17	As fast as possible for 35 secs.	c.c. per min. O ₂ 243 CO ₂ 187 R.Q. 0.76	c.c. per min. 238 181 0.76	c.c. 22230 22292 1.00	c.c. 9750 12724 1.30 T.C. 52 mins.	mgms. per cent. Before exercise 101 1 min. after exercise 101 5 mins. after 101 15 mins. after 98.5 30 mins. after 93
Jan. 25	Ditto, for 30 secs.	O ₂ 241 CO ₂ 204 R.Q. 0.84	245 197 0.80	20332 21470 1.05	7696 11070 1.43 T.C. 52 mins.	Before exercise 93 1 min. after exercise 99.5 5 mins. after 93 15 mins. after 92 30 mins. after 95

realize that the quotient cannot suddenly shift at the end of the recovery period from unity to a value characteristic of the resting period. The quotient must fall gradually along some reasonable curve. This means, of course, that as the quotient is less than unity for the last period of the recovery, it must be appreciably greater than unity at some previous time, unless the intensity of the total metabolism is so much greater at the beginning than at the end of the period that the lower quotient at the end exerts an inappreciable effect on that of the whole period.

To test this possibility, we can plot a hypothetical curve between time and respiratory quotient and slope this curve gradually towards the end of the recovery period, from unity at the beginning to the basal value found in the experiment at the end. From this curve and that of the observed oxygen consumption in the same experiment the amount of CO_2 corresponding to the quotient at various periods can be calculated. The sum of these CO_2 values should give the total CO_2 eliminated. This amount would agree with that found experimentally if the assumption that the quotient of the total metabolism never exceeds unity were correct. In a few of the experiments the calculated and the observed values for CO_2 agree reasonably well, but in most cases the latter is considerably greater than the calculated amount. This means that the respiratory quotient of the total metabolism must have been greater than unity at some period. We are, therefore, faced with the same difficulty if we consider the total metabolism as we were in the consideration of the excess metabolism, viz., the explanation of a quotient higher than unity. The experiments in Table IV illustrate very well the point we wish to make. In four of the five experiments the quotient of the total metabolism is greater than unity. It is obvious without constructing the hypothetical curve referred to above that the quotient at some period of the recovery must be greater than the value given in the table, since, as stated above, there must be a period during which the quotient gradually falls to the resting value.

The small increase observed in the total respiratory quotient in very moderate exercise indicates, we believe, that for such exercise the body is able to speed up the preparation of carbohydrate from other materials to meet the increased demand. In this case the new formation can keep pace with the rate at which the muscles consume carbohydrate. The effect on the respiratory quotient is as though any foodstuffs could be used directly to provide energy for muscular contraction. With increased severity of exercise the demand for carbohydrate increases and the process of new formation is no longer adequate to meet the demand. The increased combustion of carbohydrate may then

overshadow the effect due to the contemporary manufacturing process. Consequently we arrive at a stage where the respiratory quotient of the excess metabolism reaches unity. Finally, extremely violent exercise induces a totally new phenomenon, of which we can advance no satisfactory explanation at the present time.

Summary.

The respiratory quotient of the excess metabolism of muscular exercise is not invariably unity, but increases with the severity of the exercise from a value approximately the same as the basal quotient for very mild exercise, through unity for moderate exercise, to one considerably above unity for very severe exertion. If we disregard the excess metabolism and calculate the quotient of the total metabolism of exercise and recovery, a similar rise with increasing intensity of exercise, from values characteristic of the resting state to quotients in excess of unity, is obtained.

It is our great pleasure to acknowledge our indebtedness to Prof. A. V. Hill for his very active interest and help throughout this work. We wish also to thank Mr. Wm. Parkinson and Mr. J. L. Parkinson for their expert assistance in many of the experiments.

It is a pleasant duty for one of us (Furusawa) to thank Prof. L. M. Dennis and the staff of the Baker Laboratory for their kindness shown to him during his sojourn in Ithaca.

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The Inheritance of Sinistrality in Limnæa Peregra.

By A. E. BOYCOTT, F.R.S., C. DIVER, S. HARDY, and F. M. TURNER.

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1. The common freshwater mollusc, *Limnæa peregra*, is normally dextral: a sinistral variety, in which the spiral twist of the body and shell is completely reversed, is very rare.

2. Sinistrality behaves as a mendelian recessive character, but the appearance of any change of twist imposed by crossing is delayed by one generation. Thus a sinistral fertilised by a dextral produces (F 1) sinistral young which (F 2) produce dextral broods: these dextrals produce (F 3) dextral and sinistral broods in the proportion of 3 to 1. Similarly a dextral fertilised by a sinistral produces dextrals in F 1 and F 2 and a 3 to 1 mixture of dextral and sinistral broods in F 3.

3. Albinism in this snail is also, as usual, a simple mendelian recessive, and is inherited directly: an albino fertilised by a pigmented produces pigmented young in F 1, and F 2 consists of broods each of which contains pigmented and albinos in the proportion of 3 to 1. The characteristic shell shape of an Irish lake form of the species also disappears in F 1 on crossing with a normal specimen.

4. It is suggested that the delay in the inheritance of sinistrality is due to the fact that the twist of the animal and its shell is determined at the second - possibly at the first—division of the egg which takes place soon after the entrance of the spermatozoon. Pigmentation and shell shape on the other hand are not fixed till a later period in development and the spermatozoon would have more time to bring its influence to bear.

5. This simple scheme of inheritance is a good deal interfered with by a general tendency for sinistrals to become dextrals, some phenotypic, some genotypic.

The Free Energy of Glycogen-Lactic Acid Breakdown in Muscle.

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It is the purpose of the present discussion to show, upon the basis of thermodynamic data obtained within the last four or five years, that the free energy of glycogen-lactic acid breakdown in muscle is considerably greater than the heat of reaction, about one and one-half to two times. It is the intention to outline merely the orders of magnitude of the various quantities involved in the evaluation of this difference. This evaluation, as will be shown, need not depend upon a knowledge of the actual heat of reaction, which is still in dispute, varying between Meyerhof's value of -180 cal. and Slater's value of -235 cal. It will depend, rather, upon the specific heat differences, or ultimately, molecular structure differences, obtaining between glycogen and lactic acid. Stated briefly, the existence of this large negative difference, designated hereafter as $(\Delta F - \Delta H)$, implies that the theoretical maximum mechanical work which a muscle can perform as a consequence of this breakdown is considerably greater than the corresponding heat of reaction. The notations of Lewis and Randall (1) will be used throughout. ΔH , the heat of reaction, and ΔF , the free energy of reaction, will be negative when heat and free energy respectively are liberated.

Before presenting the thermodynamic data and calculations, it will be of historical interest to point out that in 1912 A. V. Hill (2) suggested the possibility of such a difference, when he first made the observation that during anaerobic lactic acid formation in muscle the heat evolved amounted to at least three times as much as would have been predicted if the precursor were a hexose carbohydrate. He suggested, "the breakdown from this body to lactic acid may be one of those somewhat rare but by no means unknown chemical reactions which can do more mechanical work than is equivalent to their total loss of energy; by virtue of their completeness they possess the power of absorbing heat from their surroundings to do this excess of work." Meyerhof (3, 1922) reconsidered the question, and while alive to the possibility of a considerable difference, offered an opinion, based upon the Nernst heat

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theorem, that probably no difference did exist. In general, however, little attention has been paid to A. V. Hill's original surmise, especially since further investigation of the other hydrolysis, neutralization, and deionization reactions occurring simultaneously with the formation of lactic acid, has shifted the attention to explaining the other more immediate problem, namely, the discrepancy between the observed chemical change and the required evolution of heat.

Thermodynamic Data.

Four years ago the free energies of less than half-a-dozen organic compounds were known. Since then a systematic study of the free energies of aliphatic organic compounds has been instituted by Parks, Kelley, Anderson, Huffman, and co-workers (4). The result has been not only that values for a large number of different types of organic compounds have now been obtained, but in addition, it has been found that the free energies, or more strictly speaking, the entropies, from which the free energy values are derived, are closely related to the structures of the compounds. This important finding has made possible the prediction of unknown free-energy values. So far as can be judged at present, the predictions possess an accuracy virtually equalling experimental determination, and an accuracy which, by way of relative comparison, is greater than that obtained in applying Kopp's rule to boiling points, Trouton's rule to heats of vaporization, or Dulong and Petit's or Joule's rules to specific heats. Thus the differences between the calculated and observed values of methyl, ethyl, propyl, and butyl alcohols, acetone, glycol, glycerin, formic, butyric, palmitic, and oxalic acids, and glucose are only 0.5 per cent. on an average, and are in no case greater than 1 per cent., which is about the limit of experimental determination.

The constants used to compute the entropy of lactic acid were the same as those used to calculate the entropies of the organic compounds in the preceding list, and were kindly communicated to the writer in their most revised form by Dr. K. K. Kelley. They are as follow: H, 11.3; C, -13.4; terminal O as in OH in COOH, 0.9; secondary linked O as in CHOH, -4.6; carbonyl O as in C = O in COOH, 24.4. The constants for solid compounds are a little different, since entropies of fusion are not concerned. For the entropy of liquid lactic acid, in its standard state,

$$S_{298}^0 = 6(11.3) + 3(-13.4) + 1(0.9) + 1(-4.6) + 1(24.4) = +48.3.$$

Study of the above constants will show that in addition to the fact that carbon and hydrogen always have the same values, carbonyl oxygen has an

entropy value markedly different from other types of oxygen, about 25 units. Now in any chemical reaction at constant temperature, the difference between the free energy change, ΔF , and the heat of reaction, ΔH , is given by the product of the entropy change, ΔS , and the absolute temperature, T , as follows,

$$\Delta F - \Delta H = -T\Delta S.$$

Hence an entropy difference of 25 units would correspond in any reaction to $-T\Delta S = -(273 + 25)(25 \text{ units}) = -7500 \text{ cal. per mol. at } 25^\circ \text{ C.}$ This is a large amount. Stated empirically, this means that between compounds of the same molecular formula, entropy differences will virtually be determined by the types of oxygen structures. Hence the chief differences in entropy between the compounds under discussion will depend upon the ratio of the total number of carbon atoms in the molecule to the number of carbonyl oxygens (*i.e.*, aldehyde, ketone, or oxide ring). Thus if the ratio is X in lactic acid, it is $2X$ in glucose, since the number of carbon atoms doubles, while the number of carbonyl oxygen atoms remains the same, namely, one. It is just this structural difference which is, empirically speaking, the reason for the difference between the standard free energy and the heat of reaction in the solid state. The ratio is the same in polysaccharides as in glucose, and for this reason it may be assumed, upon the basis of what may be termed the rule of Parks and Kelley, that the same equivalent standard entropy difference exists between lactic acid and unhydrated glycogen as between lactic acid and glucose.* Any standard entropy changes in the open chain "dicarbonyl" or "monocarbonyl" linkages, formed by condensation of hexoses to disaccharides and polysaccharides with the elimination of water, are not to be confused with those of the true carbonyl oxide ring linkages. The entropy changes of such open chain linkages are of a secondary order of magnitude, and will be considered quantitatively later.

It should be clearly understood that, according to the present existing thermodynamic data (4), no distinction is to be made between the various forms of carbonyl oxygen groupings. All aldehyde, ketone, and oxide ring

* It would be desirable to record an important corollary to this reasoning, suggested to the writer by Mr. John Pryde. Various structural differences between carbohydrate geometrical and stereochemical isomers, or differences such as exist between amylose and butylene oxide ring compounds, might be determined with more than the usual degree of assurance, if it were possible to detect small but definite corresponding entropy differences of, say, about one entropy unit, differences which for the purposes of this paper could be considered negligible or zero. It is not desired to imply in the discussion above, that experimental entropy differences between such isomers would be absolutely undetectable.

carbonyls possess the same experimentally determined entropy value, 24.4. All ordinarily occurring hexose carbohydrates contain one carbonyl group, and furthermore, glycogen is composed of glucose units, so that so far as the carbonyl group influence is concerned, the entropies of glycogen and glucose, per unit of carbon, are necessarily closely identical, within, let us say, 2 to 3 per cent. In connection with invariably exact, as distinguished from sufficiently approximate, quantitative support for this last statement, obviously it is to be hoped that standard entropy determinations of as many different types of carbohydrates as possible will soon be made.

The standard entropy of glucose, $S_{298}^0 = 53.4$, has been determined from the very excellent specific heat measurements made by Simon (5) over a temperature curve which ranged from 19.1° to 287.2° absolute. The calculated value using the constants of Kelley for solid substances is 53.3, a difference of only 0.2 per cent. Hence, for the transformation of solid glucose to liquid lactic acid, $\frac{1}{2}\text{C}_6\text{H}_{12}\text{O}_6$ (solid) = $\text{CH}_3\text{CHOHCOOH}$ (liquid): $\Delta S_{298}^0 = 48.3 - (53.4/2) = 21.6$; $\Delta F^0 - \Delta H_{298}^0 = -T\Delta S_{298}^0 = -(21.6 \times 298) = -6440$ cal. per mol. = -71 cal. per gram.

Granting the exactness of the two assumptions just made concerning the identity of the standard entropies of glucose and unhydrated glycogen, this means that in the reaction of pure solid unhydrated glycogen to give pure liquid lactic acid at 25°C ., the standard free energy change, ΔF^0 , is exactly -71 cal. per gram of the latter higher than the heat of the reaction in the non-aqueous state, ΔH^0 . It will be observed that, as stated before, the evaluation of this difference requires no knowledge of the actual heats of combustion. Let us now estimate how much this value will differ from the actual free energy changes under the various conditions of solution, dilution, and neutralization existing in muscle.

Solution and Dilution Processes.

We shall proceed from the standard liquid or solid states to the dissolved, diluted states. Since the heats of solution and dilution of glycogen and lactic acid are known, a certain simplification may be gained by splitting up our desired quantity and considering the resulting parts separately; thus

$$(\Delta F - \Delta H) = (\Delta F_2 - \Delta F_1) - (\Delta H_2 - \Delta H_1) - 71 \text{ cal.}$$

where ΔH_2 and ΔH_1 are the heats of dilution of lactic acid and unhydrated glycogen respectively; ΔF_2 and ΔF_1 , the free energies of lactic acid and glycogen respectively; and -71 cal. refers to $(\Delta F^0 - \Delta H^0)$ as defined and calculated above.

ΔH_2 is -14 (6), and ΔH_1 is -32 (7), so that $-(\Delta H_2 - \Delta H_1)$ is -18 . This increases our value of -71 to -89 . The accuracy of the value $\Delta H_1 = -32$, is in a sense substantiated by the fact that Slater's value (8) of -8.8 cal. for the heat of dilution of the monohydrate, agrees closely with that of Meyerhof, -10.6 cal., using a three-fold greater dilution. The probability that both investigators were using the same kind of glycogen is enhanced thereby.

Now let us for a moment consider ΔF_1 to be zero, and see how ΔF_2 , the free energy of dilution of lactic acid, will affect $(\Delta F - \Delta H)$. Fortunately we are dealing with substances which obey the laws of the perfect solution, so far as present purposes are concerned, that is to say, in terms of calories, certainly within 5 per cent. This may be seen from an examination of the water vapour pressures of their aqueous solutions. (See Ostwald (9) for vapour pressures of lactic acid solutions; Branch (10), who finds that formic acid, a monocarboxylic acid of the same strength as lactic acid, obeys the perfect solution laws very well; and Landolt-Börnstein (11), for lowering of vapour pressures of various sugar solutions.) Hence a consideration of corrections for activities may be avoided and the free energy of dilution of lactic acid may be calculated in the usual manner. Thus for lactic acid at near its maximum concentration in muscle, say 0.18 per cent. or 0.02 M,

$\text{CH}_3\text{CHOHCOOH}$ (*l.* mol. fraction ≈ 1) = $\text{CH}_3\text{CHOHCOOH}$ (aq. mol. fr. = $0.02/(55.5 + 0.02)$); $\Delta F_2 = RT \ln K = 1365 \log (0.02/55.52) = -4710$ cal./mol. = -52 cal./gm. Even at the maximum possible concentration of lactic acid in normal muscle not especially immersed in alkaline phosphate buffers (where, as Meyerhof (12, 3) has found, all the glycogen may be converted to lactic acid), let us say, 0.05 M (0.45 per cent.), this value of -52 decreases only to -49 , whereas if the concentration is reduced 10 times, to 0.018 per cent., approximately to its minimum, it increases to -67 , or if reduced 100 times, to 0.0018 per cent., below its minimum, to -82 . The lowest figure of Fletcher and Hopkins (14) for the minimum lactic acid content of resting frog muscle was 0.015 per cent. This was lower than that of any previous investigator. The most recent investigations confirm this order of magnitude, Davenport and Davenport (15) finding 0.015 per cent., Kuhn and Baur (16), 0.011 per cent., and Wacker (17), 0.010 per cent. Fletcher and Hopkins stated that the real figure is probably somewhat below even 0.015 per cent., a certain fraction of this figure being merely the result of the method of analysis. There would be no point in carrying out the calculations for still lower concentrations, however, so far as would concern any experimental contraction, where the conditions of concentration would be different from and higher than those of completely rested muscle.

This change of free energy with concentration is simply at the rate of ± 15 cal. for every ten-fold concentration or dilution respectively. Any slight disobedience of the perfect solution laws on the part of lactic acid would tend to increase the above figures for ΔF_2 , if we may judge from Ostwald's data, the activity coefficient always being less than one during dilution from the pure liquid state. However, it would require a deviation of as much as 14 per cent. less than one in order to make a difference of even $(RT/90)\ln((0.02 \times 0.86)/0.02)$, or $(1365/90) \log(0.0172/0.0200)$, or -1 cal. per gram, in the above value of -52 .

The importance of these figures for the change of free energy with concentration lies in showing that at the end of a prolonged tetanic contraction, where the concentration of lactic acid may be 0.2 per cent., the free energy is relatively only 15 cal. lower than in the case of a twitch in a fresh muscle, where the concentration is 0.02 per cent. or lower; so that when viewed absolutely, this 15 cal. is only a few per cent. of the total free energy involved, and hence possesses but small experimental interest. This means that although the heat and free energy of glycogen-lactic acid breakdown are considerably different on grounds of absolute concentration alone, even neglecting specific heat considerations, nevertheless, within the range of concentrations occurring in muscle, the differences between various free energies are comparatively small. This is indeed simplifying for the application of free energy considerations to muscle phenomena.

We have been considering ΔF_1 , the free energy of dilution of glycogen, to be zero. I am informed by Mr. W. K. Slater that 5 to 8 per cent. is the approximate maximum concentration of glycogen in water; so for the concentration of 1 per cent. which exists in muscle, $\Delta F_1 = + nRT \ln 1/5 = -n10$ cal. per gram of lactic acid, where n is the reciprocal of the ratio of the molecular weight of glycogen to that of lactic acid. Now if n , which presumably is about $1/1000$, is as high as $1/100$ even, this makes ΔF_1 only -0.1 cal. Hence ΔF_1 may be correctly considered to be zero. The low value of n similarly eliminates all quantitative significance from the possibility that the maximum concentration of glycogen may be somewhat higher than 5 per cent. (*i.e.*, 10 per cent. or 20 per cent.), owing to the particular colloidal condition it might possibly assume in the muscle plasma. We may now summarise for the reaction,

n glycogen (1 per cent.) = lactic acid (0.018 per cent.).

$$\begin{aligned} (\Delta F - \Delta H) &= -71 - (\Delta H_2 - \Delta H_1) + (\Delta F_2 - \Delta F_1) \\ &= -71 - (-14 - (-32)) + (-67 - 0) = -156. \end{aligned}$$

This gives the difference between the heat and free energy of glycogen lactic acid breakdown as it occurs in fresh unfatigued muscle, neglecting neutralization processes. Under conditions of fatigue where the lactic acid is so high as 0.45 per cent. and the glycogen so low as 0.55 per cent., it is $-(67 - 49)$ or -18 cal. less, -138 . These two values are to be compared with the -180 cal. heat of reaction for the same process occurring in muscle, so that the average free energy change is $(-156 \text{ to } -138)/-180$ or 82 per cent. (87 per cent. to 77 per cent.) higher than the heat of reaction. -180 cal. is Meyerhof and Suranyi's value (18) for the heat of reaction. Slater's value (8) is -235 . It is impossible at present to decide which is the more correct. While there seems to be no important criticism to be made of Slater's value (except that Meyerhof (38) believes that all the fat extracting solvent may not have been removed from the sample, thus resulting in a high value), Meyerhof's value agrees with that of Emery and Benedict, and lies between those of Stohmann and of Ginsberg. Slater, however, has been the only investigator to make determinations on perfectly ash-free samples of glycogen. If Slater's value for the heat of combustion of glycogen, calculated for 0.9 gm. of the anhydrous form. -386.5 , is correct, it is considerably higher than that of any other condensation product of hexoses; thus, starch and cellulose, -376 ; inulin, -372 ; dextrin, -370 ; trehalose, maltose, lactose, and raffinose, -375 (11). It is true, of course, that the same experimental criticism in regard to salt, or indeed other, impurities, may obtain for these compounds, as well as for glycogen. Meyerhof and Suranyi (18) have also found for the experimentally questionable enzymatic hydrolysis of glycogen to reducing sugars, a value of -14 cal. whereas their heat of combustion and dilution values would indicate -26 cal.; Slater's values would indicate -77 cal.

Before considering neutralization processes, let us call attention to the influence of temperature, and also reconsider a previous assumption. It will be recalled that the change of entropy with temperature is given by

$$\int ds = \int C_p d \ln T.$$

Thus if the heat capacities of diluted glycogen and lactic acid are constant over the physiological range of 0° to 40° C., or at least vary by the same amount, then change of temperature will have no effect upon $(\Delta F - \Delta H)$, both quantities changing together. Now calculation shows that even if a difference of 2 per cent. existed between the specific heat difference between the two substances at some other temperature than 25° C., say 10° C., the difference in

($\Delta F - \Delta H$) is changed only 0.1 cal. per gram. However, since the melting point of the sarcolactic or *d*-lactic acid of muscle is 25° C. (19) (whereas that of the optically inactive form is 18° C.) (20), it is possible that below 25° C. changes in specific heat differences with temperature may be somewhat larger. While there are no experimental data available to decide this point, it is conceivable that in the extreme case a difference of 10 to 15 cal. might result, in such a direction as to lower the above values.

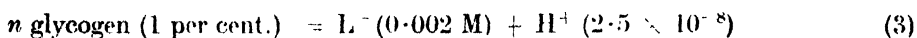
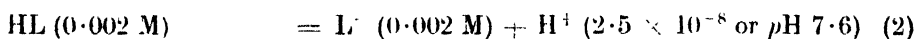
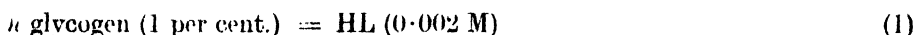
Let us reconsider the assumption that the entropies of glycogen and glucose in their solid states are equivalent. Presumably they may differ slightly. In the case of the acetic acid ethyl alcohol condensation, where conditions are objectively similar, a C-O-C grouping being formed by the elimination of water, the entropy difference corresponds to -900 cal. per mol. This would correspond to -5 cal. per gram of glucose. In their determinations of the equilibrium constants for a large number of ester formations from various common alcohols and acids, Berthelot and St. Gilles (21) found (1) that all the constants were quite generally approximately four, corresponding to a standard free energy change of -900 cal. per mol. or again -5 cal. calculated per gram of glucose; (2) that the constants were practically independent of the temperature, indicating that ΔH must be very close to zero. This latter finding may be confirmed by an examination of the probably slightly less reliable heats of combustion of the substances involved in the various esterifications, as tabulated by Landolt-Börnstein (11). In the ethyl acetate condensation cited above, $\Delta H = +200$ cal. per mol., corresponding to only 1 cal. per gram of glucose. So, to judge analogously, we should expect the entropies of glucose and glycogen to differ, if at all, in the direction slightly favouring a still greater difference in our desired quantity ($\Delta F - \Delta H$).

Fortunately, in addition to the above condensations, one carbohydrate equilibrium has been investigated experimentally, and conclusions drawn from consideration of it will be even more pertinent to the establishment of the correctness of the above assumption. Calculations of the writer show that the standard entropy of the glucose-maltose condensation is, in fact, less than that cited above for the acetic acid ethyl alcohol condensation, the experimental error being about the same in both cases. Using the heat of combustion data of Stohmann (11), and recalculating the equilibrium data of Croft Hill ('Trans. Chem. Soc.,' vol. 73, pp. 634-658 (1898)), ($\Delta F^0 - \Delta H^0$), or $-T\Delta S^0$, is only -300, rather than -900 as above. The absolute experimental error in both these cases may, of course, amount to 1000 cal., but, obviously, even this is more than sufficiently accurate for the purposes at hand.

Finally, so far as the writer knows, there is no reason for supposing that the standard entropies of all the various ester, glucose, and glycogen condensations are not of the same order of magnitude and close to zero. It might be urged that comparison between the first two and the last is not strict, since experimentally the former are freely reversible, while the latter is not. However, it must be recalled that free reversibility is not a function of the magnitude of entropy change, only of the magnitude of free energy change, which in various condensations may or may not be of the same order. It is quite possible for reactions, in which the entropy change is zero, to be highly irreversible, owing to a large free energy change. A typical instance is the combustion of graphite, where $\Delta F^0 = 94260$, $\Delta S^0 = 0$.

Neutralization Processes.

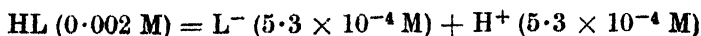
The free energy of neutralization of lactic acid depends upon the hydrogen ion concentration, or, in other words, the degree to which the neutralization is carried out. The problem is one of calculating the free energy liberated by the neutralization of the hydrogen ion created by lactic acid introduced into a pre-existing system of phosphate, carbonate, lactate, and proteinate already in equilibrium at the pH of muscle plasma. It is treated in the simplest manner by splitting our finally desired reaction (3) into its two components, reaction (1), which we have already considered, and reaction (2), which we shall now consider.



Now the dissociation constant of lactic acid is 1.4×10^{-4} , so that at a concentration of 0.002 M undissociated lactic acid,

$$K = 1.4 \times 10^{-4} = \frac{(5.3 \times 10^{-4} \text{L}^-)(5.3 \times 10^{-4} \text{H}^+)}{2 \times 10^{-3} \text{HL}}; \Delta F = 0.$$

This is to say, at a concentration of 0.002 M undissociated lactic acid, the reaction



represents the equilibrium condition where the free energy of neutralization is zero. Therefore, under the conditions of muscle plasma, where we assume a pH of 7.6 (*i.e.*, $\text{H}^+ = 2.5 \times 10^{-8}$) and $\text{L}^- = 0.002 \text{ M}$ (calculation shows

that only about 0.3 per cent. of the undissociated HL exists at pH 7.6, namely, 3.5×10^{-7} M HL), the free energy of reaction (2) above is

$$\begin{aligned}\Delta F &= RT \ln \frac{2 \times 10^{-3}}{5.3 \times 10^{-4}} + RT \ln \frac{2.5 \times 10^{-8}}{5.3 \times 10^{-4}} \\ &= -5090 \text{ cal./mol.} = -57 \text{ cal./gm.}\end{aligned}$$

Similarly, at pH 6.9 (*i.e.*, $H^+ = 1.3 \times 10^{-7}$)

$$\begin{aligned}\Delta F &= RT \ln \frac{2 \times 10^{-3}}{5.3 \times 10^{-4}} + RT \ln \frac{1.3 \times 10^{-7}}{5.3 \times 10^{-4}} \\ &= -4110 \text{ cal./mol.} = -46 \text{ cal./gm.}\end{aligned}$$

Before comparing these values with the heat of neutralization, let us observe that they are independent of the dissociation constants or strengths of the neutralizing agents, whether these be phosphate, carbonate, or proteinate. This is true since at any given pH, the concentrations, or more strictly speaking, the partial molal free energies, of these agents remain constant (1, pp. 41, 291-3; 22, pp. 304-6). Even when the pH changes by a few tenths of a unit, the partial molal free energies change negligibly, since they are logarithmic, rather than direct, functions of the hydrogen ion changes involved. Thus, when the pH of a solution of phosphate is changed from 7.4 to 7.6, the proportion of $(HPO_4)^-$ is changed only 6 per cent. In terms of partial molal free energies, this is $RT \ln 1.06 = 34 \text{ cal./mol.} = 0.3 \text{ cal./gm.}$ of $(HPO_4)^-$. Such a refinement in regard to the change in the proportions of dissociated and undissociated forms of the neutralizing agents has therefore been neglected in the above calculation.

The -57 cal. at pH 7.6, and the -46 cal. at pH 6.9, are to be compared with the heats of neutralization of lactic acid by monohydrogen phosphate of -19 cal. and by proteinate of -140 cal. Hence in muscle whether the free energy is greater or less than the heat of neutralization depends upon the proportion of protein to phosphate or other buffering substance concerned. This proportion will depend upon the concentrations and dissociation constants, the larger the concentration or constant, the larger the amount combined. The apparent acid dissociation constant of muscle protein is not known. Presumably it could be determined by distribution studies. There might be good reason to assume with Meyerhof (3, p. 62) that it is about 10^{-6} , since the isoelectric points of most proteins are slightly more acid than pH 6. The order of Meyerhof's value is confirmed by the measurements of the apparent acid dissociation constants of various muscle plasmas made by Furusawa and

Kerridge (23), who find the constants slightly smaller than 10^{-6} , owing, no doubt, to the averaging influence of the still smaller constants of the phosphate, 2×10^{-7} , and bicarbonate, 3×10^{-7} . However, let us consider that as determined experimentally by Meyerhof and Lohmann (24, 18), 7/14 to 9/14 or an average of 8/14 of the lactic acid reacts with protein to give $(8/14 \times -140)$ or -80 cal., and that the rest reacts with phosphate to give $(6/14 \times -19)$ or -8 cal., a total of -88 cal. In this connection, the newly discovered pyrophosphate (25) is equal in concentration and neutralizing power to the ordinary monohydrogen phosphate, in the pH range under consideration. Furthermore, the phosphate set free from phosphagen is available. Therefore, under these conditions, the free energy of neutralization (-57) is 31 cal. less numerically than the heat of neutralization (-88). If the neutralization were accomplished entirely by phosphate it would be 38 cal. more than the heat of neutralization, but if accomplished entirely by proteinate, it would be 83 cal. less. We may explain these very different values which obtain, whether lactic acid is neutralized by proteinate or phosphate, as being caused by the anomalously high deionization heat of proteinate. Even in the case of ordinary acids, carbonic, diphosphoric, etc., with similar dissociation constants of about 10^{-6} to 10^{-7} , the deionization heat is very small, *i.e.*, about -1000 to -3000 cal. per mol., rather than -12500 . This anomalous condition constitutes the chief ground we might have for expecting the apparent acid dissociation constant of muscle protein not to be 10^{-6} , but similar to those of other acids, such as amino acids and hydrocyanic acid, which have very much smaller dissociation constants of about 10^{-10} , and deionization heats of -10000 cal. or more. One should hardly expect Berthelot's principle to fall into such great error.

The final value of $(\Delta F - \Delta H)$ for reaction (3) above, including neutralization processes, is therefore $(-156 - (-31))$, or -125 cal. This means that the free energy is $(-125 - 268)/-268$ or 1.46 times as great as the heat of reaction.

It will reward us to call attention to the case of strong acids and bases. When these react at concentrations, or more strictly speaking, activities, of one molal, the free energy is -19105 . This is -5400 cal. more than the heat of neutralization, -13700 . Calculation shows that the free and heat energies do not become equal until the logarithm of the product of the concentrations of OH^- and H is increased $(-13700 - (-19105))/2.3 \text{ RT}$ or $5400/1365$ or 3.96 times. This means, if both are reduced equally, to concentrations of 0.01 M. When a molal solution of a strong acid is neutralized

by a buffered solution at pH 7, the free energy is only -9555 . The heat of neutralization in such a case would also, of course, be less than -13700 , by an amount equal to the heat of ionization of the buffering substance.

Complete Anaerobic Balance Sheet.

According to Meyerhof's latest balance sheet, the heat of glycogen-lactic acid breakdown and the heat of neutralization of lactic acid (distinguishing between the two in this case merely for the sake of clarity) account for only -260 to -280 of the -380 cal. observed, leaving -100 to -120 cal. still to be accounted for by other chemical reactions. Using Slater's values, these would be reduced to -50 to -70 cal. The dehydration of protein, the hydrolysis of phosphagen, and the formation of ammonia have been three other suggested reactions, but there is little experimental support for them at the present time: indeed, rather the contrary (see Nachmansohn, 26). Since we shall have to know all the chemical reactions concerned in contraction before we can determine ΔF , and hence $(\Delta F - \Delta H)$, for all processes, we might not be entirely justified for the present in assuming, upon the basis of the data which have been presented, that for the anaerobic contraction as a whole, ΔF is greater than ΔH . However, so far as these dehydration and hydrolysis reactions are concerned, as we have seen above, they are of a type in which $(\Delta F - \Delta H)$ is small. Furthermore, during very short periods of stimulus, about 0.1 sec. or less, the value of $\Delta H = -380$ cal. may be too large at this region of length of stimulus, where it cannot be measured directly in combination with chemical means.

It is to be remembered that so far as the explanation or partial explanation of this discrepancy is concerned, particularly where very short stimuli are concerned, there is as yet no need to resort to reactions entirely foreign to glycogen-lactic acid breakdown, since knowledge of the changes undergone by intermediates is incomplete. Although, as Meyerhof has shown (27), the lactic acid formed approximately equals the glycogen consumed, this stoichiometric relation does not preclude the possibility that the concentrations of the intermediates change with respect to each other. So long as the concentrations of at least *two* of the intermediates change, and change in directions of opposite sign, the stoichiometric relation is entirely possible.

Furthermore, it should be clearly understood that so far as comparing the observed and calculated values for the heat evolved per gram of lactic acid formed over a relatively long period of stimulation or time, no consequences are entailed by the difference between the heat and free energy. Under these

circumstances, the process can be considered as entirely irreversible, all the potential energy being converted into heat. On the other hand, if one were to compare the time courses of tension and heat in a twitch or short tetanus, in an attempt to assign clearly defined and differentiated chemical causes to the heats of development and maintenance, after the manner, say, of Garner (28), Meyerhof (29, p. 78), A. V. Hill (30, 31), or Tiegs (32), then, obviously, any tension set up might tend to cool the muscle somewhat, and a positive correction to these heats might be necessary.

Aerobic Breakdown.

In the aerobic muscular process, the recognisable end product is not lactic acid but carbon dioxide, and as the following figures show, in this case there is no important difference between the heat and free energy. In the anaerobic process it is only because the heat energies involved are so small, that the free energies can differ so considerably from them. The heats of combustion of carbohydrates are so large that entropy factors become negligible, in comparison.

The standard free energies of formation of CO_2 and H_2O are -94260 and -56560 , the respective heats are -94250 and -68270 (4). Using the entropies cited previously, the standard molal free energies of combustion of liquid lactic acid, solid glucose, and glycogen (per $\text{C}_6\text{H}_{12}\text{O}_6$), are -325160 , -685800 , and -699900 , if the heats of combustion are taken to be -325720 (6), -674000 , and -688100 (7). The respective values for $(\Delta F - \Delta H)$ in the standard states are 560 , -11800 , and -11800 , or differences of 0.2 per cent., -1.7 per cent. and -1.6 per cent. Under atmospheric conditions the respective values for $(\Delta F - \Delta H)$ are -7590 , -29300 , and -38900 , or differences of -2.5 per cent., -4.5 per cent. and -5.7 per cent. Here the CO_2 is considered to be at 0.0003 atmos., O_2 0.2 atmos., and the concentrations of the substances, lactic acid 0.18 per cent., glucose 1 per cent. (and its maximum solubility in water 90 gm./ 100 gm. water so that its molal free energy of dilution is -2700), and glycogen 1 per cent. The heats of combustion have been taken, correspondingly, as -324460 (6), -677000 (33), and -682400 (7). These small percentage differences for aerobic processes should be compared with the value of 87 per cent. for anaerobic processes. If the O_2 and CO_2 are at tensions corresponding to those in blood, $1/10$ and $1/20$ atmospheres, respectively the values for $(\Delta F - \Delta H)$, and for the percentage differences, are practically the same as those given first for the standard states rather than those of the second group.

Intermediate Product Breakdown.

We must discuss in some detail the possibility that whereas glycogen-lactic acid breakdown may be the chief reaction of energetic importance occurring in a long tetanic contraction, nevertheless, in the case of a single twitch or short tetanic contraction, the heat and tension may be developed so rapidly that decomposition of glycogen lags, the very immediate chemical reaction being the breakdown to lactic acid on the part of some such intermediate compound as hexosephosphate. The following reasoning and experimental evidence do not credit this possibility with important qualitative significance for the energy calculations.

We may regard the occurrence of a large negative value of $(\Delta F - \Delta H)$ in any case as owing not so much to the nature of any parent substance, as to those two unusual properties characterizing the common end-product lactic acid, namely, (1) a ratio of as many as two carbonyl groups per $(\text{CH}_2\text{O})_6$, and (2) an infinite or very great solubility in water. Indeed, we may make the fairly general statement that for $(\Delta F - \Delta H)$ to be close to zero, the intermediate must possess both these properties. Neither glycogen, hexosephosphate nor glucose, do, nor is it conceivable that any other carbohydrate with at least six carbon atoms per molecule would do so either. So much for the smallest value of $(\Delta F - \Delta H)$. Now let us consider its highest value.

$(\Delta F - \Delta H)/\Delta H$ was found to be 87 per cent. for glycogen-lactic acid breakdown (neglecting neutralization processes for ease in comparison). $(\Delta F - \Delta H)/\Delta H$ for glucose-lactic acid breakdown, when the concentration of glucose is 0.1 per cent., is only 40 per cent., when entirely comparable calculations are carried out. While the concentration of glucose in muscle is nearer 0.01 per cent., 0.1 per cent. has been considered in order to include, in a general way, hexoses bound as hexose-phosphates. Now, for all compounds with one carbonyl oxygen per $(\text{CH}_2\text{O})_6$, we may regard 87 per cent. and 40 per cent. as the limiting high and low values of $(\Delta F - \Delta H)/\Delta H$, since it so happens that (1) glycogen has (a) a very small or negligible free energy of dilution, (b) a large negative heat of dilution (-32), and (c) a high concentration in muscle; whereas glucose has the reverse (a) a very large free energy of dilution (-45), (b) a large positive heat of dilution (30), and (c) a low concentration in muscle. The three circumstances in the case of glycogen are therefore very favourable to a nearly maximum value of $(\Delta F - \Delta H)/\Delta H$, in the case of glucose to a nearly minimum value. Hence for a value in the case of some other intermediate to obtain outside the limits 40 per cent. to 87 per cent. would require

a still more fortuitous and hardly conceivable combination of these same circumstances.

Comparison of Observed and Calculated Free Energies.

In view of new experimental information concerning observed muscle efficiencies which promises to appear during the next year or two, it appears desirable to delay a presentation of quantitative comparisons of observed and calculated free energies in connection with determining the free energy efficiencies of muscular contraction. The method of measuring the observed free energy (*i.e.*, the theoretical maximum work) is at present undergoing modification. In a paper by Hartree and A. V. Hill to appear shortly, the manuscript of which was kindly shown to the writer, the view receives support that the truest measure of the mechanical potential energy is the heat liberated in relaxation, rather than the tension length diagram area as used by A. V. Hill (35) and others before. Such a view, for which Meyerhof (38) has always contended, upon other grounds, however, has received this new support as a result of further investigation of the Fenn effect (36, 37). The determination of the theoretical maximum work is further complicated and rendered uncertain by the possibility suggested by A. V. Hill (35, p. 261, lines 18–21) and later by Wyman (39), that part of the potential energy of tension may be used in reconversion processes rather than all being converted quantitatively into heat. Now, although the new data of Hartree and A. V. Hill present unexpected evidence against such a possibility, and Meyerhof (38) rejects it on certain *a priori* grounds, the suggestion is, in fact, not to be turned aside lightly. We must remember that the total molal energies involved in reconversion are so small that the reactions are thermodynamically quite reversible—equilibrium factors are determining ones—potentially at least. The precise determination of both the existence and magnitude of such a reconversion is not only of importance in connection with the problem at hand, but also for the interest which would attach itself to such an unusual instance of the conversion of mechanical into chemical energy.

The free energy efficiency of aerobic contraction, be it observed, is more easily ascertained than that of anaerobic processes, since for one thing, the free energies are approximately the same as the heat energies. Probably the final values for the aerobic kinetic energy efficiencies of ordinary mammalian muscle will always be found to be near those limits first determined by Joule and Thomson (48), namely, 15 to 25 per cent. We may expect a greater variation in the anaerobic efficiencies, however, and in extremely favourable

cases 80 to 90 per cent. of the energy liberated will possibly be found to pass through the mechanical, or free, energy stage. The whole problem of making anaerobic comparisons is complicated at present (although no doubt one day it will be simple enough), and easily formed tentative conclusions are to be treated with reserve. Therefore new data will be awaited with great interest.

Finally, we must mention the implications of the second law of thermodynamics enunciated by Carnot (40), extended by Clausius (11), and defined in terms of probabilities by Gibbs (42) and Boltzmann (43). Just as in any consideration of heat energies, the most important tacit assumption is the first law of thermodynamics, so, in any consideration of free energies, the most important tacit assumption is the second law of thermodynamics. If this law is applicable to biological forms considered as machines capable of utilizing chemical reactions to accomplish any of the various forms of "work"—potential, mechanical, electrical, chemical—then such biological machines must in no case yield more free energy than they receive. Although theoretical reservations, of substantial character, as to the law's applicability to animate matter have been suggested by Kelvin (44), Wand (44A), Helmholtz (45), and Donnan (46), the present possible tentative comparisons, to be refined upon the basis of forthcoming data and given in a later paper, will be seen to support this law so far as might be expected from the security of the data from which they are derived: regardless of the method used to determine the experimental physiological values, these are always smaller than the calculated maximum thermodynamic values. Such tentative comparisons are perhaps little more adequate than those of Parker (47), who made the first serious attempt to prove the biological applicability of the law, in this instance to photosynthesis. However, the writer will later give a proof, accurate to about 1 per cent., of the law's applicability to those autotrophic bacteria which oxidize hydrogen and reduce carbon dioxide simultaneously, the thermodynamic free energy efficiency of the process, which in this case involves no mechanical energy, being 100 per cent.

For the more recent discussions of the applications of free energy considerations to living phenomena, reference may be made to (2, pp. 503, 507), (3), (35, p. 257), (46), (49), (50), (51), (52), (53), (54), (55), (56).

Summary.

It has been the task of this paper to present a discussion of certain of the free energy relations of chemical and physical processes occurring in muscle.

It is shown that the free energy of anaerobic glycogen-lactic acid breakdown in

muscle is one and one-half to two times as great as the corresponding heat of reaction. This relation depends chiefly upon two physical factors—specific heat and solubility differences between glycogen and lactic acid. For aerobic glycogen-carbon dioxide breakdown, the free energy is only 1 to 6 per cent. greater. This means that the maximum mechanical work which a muscle might perform, as a consequence of anaerobic breakdown, is considerably greater than corresponds to the heats of the causal chemical changes, the extra heat energy being derived from the surroundings; for aerobic breakdown, it is closely the same, however.

The discussion given has emphasized how essential a knowledge of the free energies, as distinguished from the heat energies, is in determining which chemical reaction is chiefly responsible for the development of mechanical energy in muscle; the molal heat energies are small, and equilibrium or entropy factors become determining ones. In view of the high free energy values found to obtain above, the chief energetic importance of glycogen-lactic acid breakdown, which assumes for itself practically the whole of the responsibility for the development of mechanical energy in muscle, receives fresh, independent, support.

I take much pleasure in acknowledging helpful criticism given by Prof. A. V. Hill, Mr. W. K. Slater, Prof. W. E. Garner, Prof. O. Meyerhof, and Dr. K. Lohmann. In addition, I wish to express appreciation of the numerous courtesies extended by Prof. J. C. Drummond during my year's stay in his laboratory.

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*The Functions of the Corpus Luteum.—I. The Mechanism of
Œstrus Inhibition.*

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[PLATES 2 & 4.]

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I.—*Introduction.*

The methods by which the action of the corpus luteum can be studied have recently become greatly extended. It is possible to eliminate the corpora lutea by X-ray sterilisation, while, on the other hand, the production of luteal tissue may be intensely stimulated by the administration of anterior pituitary extracts. It is also possible to obtain extracts of corpora lutea which will substitute at least one function of the corpus luteum. As test objects, the vaginal smear test for œstrus inhibition, the placentoma test for the sensitization of the uterus, and the experimental study of the rabbit mammary gland, provide easy means of ascertaining luteal activity. It is intended, in the series of reports of which the present is the first, to record an investigation into the physiology of the corpus luteum carried out by means of these various techniques.

In previous papers (Parkes and Bellerby, 10, 11) the evidence that the corpus luteum has an œstrus-inhibiting action was fully discussed, and a method of preparing an œstrus-inhibiting extract of the organ was described. In the present paper various lines of work bearing on this problem will be recorded

It has been pointed out before (Parkes, 8) that, since obliteration of the corpora lutea in the ordinary unmated cycle of the mouse does not affect the length, the corpora lutea under these conditions can have neither an œstrus-promoting nor an œstrus-inhibiting action. After sterile copulation, however, the corpora lutea of ovulation persist and the interval before the next œstrus period is prolonged from 4 days to about 12 days. In this paper it is hoped to be able to demonstrate the dependence of the latter upon the former event.

During lactation, also, when a full-sized litter is being suckled, the corpora lutea of the immediate post-partum ovulation persist and œstrus is in abeyance. It has been shown (Parkes and Bellerby, 10) that the injection of several mouse units of œstrin is necessary to overcome the œstrus-inhibition set up by lactation, and it was demonstrated at the same time that this inhibition was dependent upon the presence of the ovary. In this paper it is shown that not only is the inhibition dependent upon the presence of the ovary, but in particular upon the presence of the corpora lutea.

II.—*Methods and Material.*

Albino mice have been used exclusively for the experiments described. Bilateral and unilateral X-irradiations have been performed as previously described (Parkes, 9). Operative technique, detection of œstrus, etc., have also been as usual (Parkes, Brambell and Fielding, 12; Parkes, 7).

The sodium hydroxide extracts of anterior pituitary have been prepared from the directions given by Evans (4) and by Teel (15). The dose used was 1.0 c.c. per day, equivalent to approximately 1 gm. original tissue.

The suspensions of anterior pituitary have been made by macerating the whole of a male mouse gland in saline, and injected subcutaneously.

Serial sections were made of the ovaries of all mice discussed in this paper. The ovaries were fixed in Bouin's fluid; the sections were cut at 7 μ and stained in Erlich's hæmatoxylin and eosin.

III.—*Pseudo-pregnancy.*

The inhibition of œstrus and ovulation which follows sterile copulation in the mouse and rat is known to be associated with the prolongation of the functional life of the corpora lutea of ovulation. It would be expected, therefore, that the elimination of ovulation would result in sterile copulation taking place every 4 or 5 days, *i.e.*, at the periodicity of œstrus in the unmated female. Two sets of material bearing on this point are available.

(a) *Untreated Non-ovulating Mice.*—Three untreated mice were observed in

which sterile copulation took place with the periodicity characteristic of œstrus in the unmated animal.

AM1 copulated four times while under observation, and in each case the interval was 3 days. Autopsy was made one day after the last copulation. Histologically, the ovaries were found to contain a few small corpora lutea, but these were atretic and in most the remains of the ova were present (Plate 2, fig. 2). A group of large follicles was also present. These were all between 600 μ and 700 μ in diameter. This is larger than the immediate pre-ovulation follicle of the normal mouse (Brambell and Parkes, 1) and is entirely abnormal for the day after ovulation. From this large size and also from their histological appearance it is probable that these follicles were becoming cystic, and in any case it is obvious that ovulation and the formation of true corpora lutea was at least temporarily suspended in this animal.

AM5 copulated three times at four-day intervals. Four days after the last occasion the left ovary was removed. Ten days later a further copulation occurred and pregnancy supervened. The ovary removed at operation showed atretic corpora lutea and one or two atretic follicles, but no large follicles such as were found in AM1 were observed. In this animal, therefore, the suspension of ovulation appears to have been brought to an end as a result of the unilateral ovariectomy, or for some other reason shortly after the operation.

AM6 copulated three times, the intervals being 5 days and 2 days. The left ovary was removed 8 days after the last copulation. Conception occurred on the last occasion, but it is evident that two periods of œstrus attended by copulation were unaccompanied by ovulation, and therefore not followed by pseudo-pregnancy.

These observations well support the idea that pseudo-pregnancy is dependent on the presence of corpora lutea.

(b) *Experimental Elimination of Corpora Lutea.*—In previous papers (Parkes, 8) the effect on the œstrous cycle of the mouse of eliminating follicles and corpora lutea has been discussed, but no adequate investigation into the occurrence of pseudo-pregnancy after sterile copulation in the sterilised animal was made. In an endeavour to throw light on this problem a series of 60 adult animals were mated with vasectomised bucks, and when the length of the cycle after sterile copulation had been established for the series, a sterility dose of X-rays was given. The results were, however, most contradictory. In a small percentage of cases the theoretical expectation of copulation every 3 to 5 days was realised, but in most of the animals the cycle, after a short period of normality, became very erratic, in some cases fading out altogether.

Histologically, definite differences were found between the ovaries of the animals copulating regularly and those in which the cycle became erratic. The former were similar to the ovary typical of the unmated sterilised female, while the latter all tended to be of the type which has previously been described as luteal (Brambell, Parkes and Fielding, 2, 3). Since copulation is known to stimulate the luteal tissue in the normal animal, it may be supposed that copulation under certain conditions tends to bring about the luteinisation of the sterilised ovary, and that this luteinisation is responsible for the disturbance of the cycle. This supposition becomes probable when considered in relation to the experiments described below on the luteinisation of the X-rayed ovary by anterior pituitary extracts.

IV.—*Lactation.*

In a previous paper (Parkes and Bellerby, 10) it was shown that the ovary during lactation has a considerable œstrus-inhibiting power, and it was concluded that this effect was produced by the persistent corpora lutea of lactation which occur in the mouse. It has now been possible to confirm this supposition. A technique has recently been described (Parkes, 9) which makes possible the elimination of the corpora lutea without interfering with the functions of the ovary, other than those performed by the corpus luteum. This technique consists of sterilising one ovary of the young mouse and then, at the required stage, removing the ovary containing the corpora lutea; the irradiated ovary, capable of producing œstrus, etc., being left. In the experiments to be described unilaterally sterilised mice were allowed to become pregnant and to rear their litters. Shortly after parturition the left (fertile) ovary was removed, and at 10 to 12 days post-partum a small dose of œstrin was given. The results are shown in Table I.

These results show that after ablation of the corpora lutea, leaving intact the other functions of the ovary, a small amount of œstrin is sufficient to produce œstrus during lactation. This is quite contrary to what was found when corpora lutea were present, and corresponds to what was found after double ovariectomy of the lactating mouse. It seems evident, therefore, that the œstrus-inhibiting activity of lactation is exerted solely through the corpora lutea which are directly or indirectly caused to persist.

Table I.—Effect of Injection of Œstrin during Lactation after Removal of Corpora Lutea.

Number of animal.	Days lactating at operation.	M. U. Œstrin given.	Number suckling.	Condition of right ovary.	Result.
29	6	0	7	Fertile	Negative
40	5	0	5	Sterile	"
41b	4	1	6	"	"
43	5	1	7	"	Positive
44	3	2	4	"	Negative
45	2	2	8	"	Positive
55	5	1	6	"	"
69	6	1	6	Fertile	Negative
70	7	2	6	Sterile but corpora lutea	Positive
74	7	2	6	Sterile	"
75	5	2	5	Fertile	"
78	7	2	5	Sterile	"

V.—Administration of Anterior Pituitary Preparations.

Remarkable effects on the ovary of the injection of anterior pituitary preparations have recently been described. Evans (4, 5, 6) and Teel (15) have found that the injection of NaOH extracts of ox anterior pituitary tissue had the effect of causing all large follicles in the rat ovary to form luteal tissue. The corpora lutea formed were atretic, in so far as they were formed without the intermediate act of ovulation, but they differed from ordinary atretic corpora lutea in being remarkably healthy histologically, and also in being functionally active, both as regards œstrus-inhibiting properties (Evans, 4, 5, 6) and the sensitization of the uterus to mechanical irritation (Teel, 15). More recently Smith and Engle (13, 14) have described the action on the mouse ovary of implantation of macerated anterior pituitary tissue. These authors found that this treatment resulted in the maturation of very large numbers of follicles and in "super-ovulation." Similar effects have been noted by Zondek and Aschheim (16, 17). In view of the distinction between the two types of result, (a) the luteal stimulating action, and (b) the stimulus to follicular maturation, it seems possible that two distinct factors regulating ovarian activity may be located in the anterior pituitary body.

Since the action of the NaOH extract would seem to depend on the presence of follicular tissue, it was decided to investigate the action of the extract on

X-irradiated mice from which the follicular tissue had been eliminated. The following experiments were therefore undertaken :—

- (a) Administration of the NaOH extract to normal female mice.
- (b) Administration to sterilised mice of the NaOH extract and also of the tissue suspension.

(a) *Normal Adult Mice*.—Twenty-four mice in all were injected with the NaOH extract. The details are summarised in Table II.

AP28–30–32 had the left ovaries removed 9 days after the first injection, order that two experimental stages could be secured from each animal.

Table II.—Injection of NaOH Extracts of Anterior Pituitary to Normal Female Mice.

Number of animal.	Injections begun, days after oestrus.	Days injected.	Days diestrus.	Notes.
AP1	3	4	7-	
AP2	3	9	13+	
AP3	1	8	6	
AP4	On oestrus	7	7-	
AP5	7	7	15	Normal cycles later.
AP6	On oestrus	4	4+	Died.
AP7	7	3	—	"
AP8	2	3	6+	
AP9	4	3	17+	Ill when killed.
AP10	1	3	4+	
AP11	1 day before	2	—	
AP12	1	3	12	
AP22	On oestrus	15	14+	
AP23	4	15	19+	
AP24	On oestrus	15	15-	
AP25	3	15	29	Normal cycles later.
AP26	On oestrus	15	19	Became pregnant at next oestrus.
AP27	..	15	22+	
AP28	..	9	14;	
AP29	..	9	9	Pregnant at next oestrus.
AP30	..	9	9	" "
AP31	..	9	12	" "
AP32	..	9	8+	Died.
AP33	..	9	21+	

Histological Effects on the Ovary.—Ovaries examined after 7 to 10 days injection show complete or practically complete absence of follicles beyond the stage of the appearance of the antrum. Younger follicles than this are still to be found (Plate 2, fig. 1). Small, but healthy, corpora lutea are present in great numbers, up to 40 to 60 in one ovary. In the majority of these the remains of the ovum may be found (Plate 2, fig. 3), proving conclusively that

these corpora lutea are not formed as the result of ovulation. The remains of the ovum may be relatively obvious, or, in a few cases, difficult or impossible to find. The less crowded and the younger corpora lutea show their origin most clearly.

The first stage in the formation of these corpora lutea atretica is characterised by the swelling up of the follicular epithelium, with a corresponding crushing in of the antrum. Subsequently, the ovum shows atrophic changes, such as fragmentation of the nucleus and general shrinkage. The hypertrophying follicular epithelium gradually closes in on the remains of the ovum and a solid body is formed (Plate 2, fig. 4). Mitosis was not observed during this process, and the increase in the amount of tissue is accounted for solely by the swelling (largely of the cytoplasm) of the individual cells. Allowing for the restricted space, and the presence of liquor folliculi and ovum, the process appears to be analogous to that occurring normally after ovulation.

The result of these changes is that an immense amount of luteal tissue is formed in the ovary (Plate 2, fig. 1). The atretic corpora lutea are crowded closely together, without, however, becoming confluent. They remain as discrete bodies with perfectly definite lines of demarcation.

Effect on Œstrus. In nearly all animals no signs of Œstrus whatever were observed during the time the injections were being made, but in a few where Œstrus was due on the first or second day of injection, its occurrence was not inhibited. Since it has already been shown (Brambell and Parkes, 1) that Œstrus will occur up to 2 days after double ovariectomy, and, therefore, that the Œstrus-producing stimulus becomes operative at least 2 days before Œstrus symptoms appear, the occurrence of Œstrus up to 2 days after the beginning of the injections was not surprising. The fact that it did not occur more often suggests that the anterior pituitary extracts have a very strong luteal stimulating action.

Many of the animals were killed before the induced dioestrus had concluded and its full length was not, therefore, ascertained in all cases. Table II, however, makes it clear that the duration and intensity of the reaction was very irregular. This is not surprising considering the crudity of the method of preparation, and the probable variation in the activity of the extracts.

In seven animals in which the data are available, the time of reappearance of Œstrus after the last injection was 1, 1, 11, 5, 4, 6, 4 days. In the first two, however, one extract has been used continuously for 7 to 8 days and had probably become inactive. The last five figures, therefore, give a truer idea

of the hiatus between the last injection and the reappearance of œstrus. The last four of these animals copulated at the first œstrus after the recommencement of the cycle and became pregnant. Ovulation, therefore, starts again at the same time as the changes in the accessory organs. Six mated animals all failed to copulate during the time injections were being made.

(b) *X-irradiated Females*.—The first injections into sterilised female mice were made in the expectation that since the sterilised ovary contained no follicular tissue, no luteal tissue would be formed and no inhibition of œstrus would occur. It immediately became obvious, however, that this expectation would not be realised. All sterilised animals injected with the NaOH extract of anterior pituitary immediately went into diœstrus, and showed no further cyclic activity during the time the extracts were being injected. Some in fact failed to show œstrus for a prolonged period after the end of injection, and it was at first thought that the œstrus inhibition might be permanent. Certain animals, however, did finally show a return of œstrus. Histological examination showed that remarkable changes had taken place in the ovaries of the injected sterilised females.

The details of this series of animals are given in Table III.

Table III.—Injection of NaOH Extracts of Anterior Pituitary into X-irradiated Mice.

Number of animal.	Days injected.	Days diœstrus.	Return of œstrus after last injection.	Time of left ovariectomy after beginning of injection.	Time of autopsy after first injection.
			Days	Days	Days
APX33	15	14 +	---	---	15
APX34	15	15 +	---	---	15
APX35	15	15 +	---	---	15
APX36	15	21	8	---	26
APX37	15	24	11	---	28
APX38	15	19+	---	-	21
APX42	3	0	---	0	2
APX43	9	12 +	---	0	8
APX44	9	14	7	0	16
APX47	9	21+	---	0	23
APX48	9	18	11	0	23
APX50	9	12	7	0	16

Histological Effects on the Sterilised Ovary.—The usual type of ovary resulting from X-irradiation at 3-weeks old has been adequately described elsewhere (Brambell, Parkes and Fielding, 2, 3), together with the occurrence of œstrus after the obliteration of the follicular system. In a small percentage of cases,

however, an abnormal type of sterilised ovary was found. The sterilised ovary consists largely of tissue proliferated from the germinal epithelium, and the abnormal type of this tissue, instead of being of a parenchymatous nature and œstrus-producing as is usual in the sterilised ovary, becomes most definitely luteal like. This abnormal luteal type of sterilised ovary was found to be associated with the suppression of œstrus.

It was found that the injection of NaOH extracts of anterior pituitary into sterilised animals had apparently brought about the luteinisation of the sterilised ovary in every case. Animals injected up to the time of autopsy had ovaries consisting mainly of typical luteal tissue. In most cases this was in the form of large ill-defined patches, merging with each other at various points. APX35 is an example of this type (Plate 3, fig. 1. and Plate 4, fig. 1). In extreme cases the luteinisation appeared to be almost complete and the whole ovary, allowing for topographical differences, resembled one large corpus luteum. The individual cells were large and remarkably healthy looking (Plate 4, figs. 2, 3, 4). Since all animals used for this experiment were first observed to exhibit cyclic œstrus, it is clearly impossible, quite apart from considerations of probability, that these ovaries could originally have been of the luteal sterilised type.

This extraordinary effect on the non-follicular and histologically non-cyclic tissue of the sterilised ovary is clearly analogous to the luteinisation of the follicular epithelium of the normal animal by the same treatment, and is presumably made possible by the common origin of the two in the germinal epithelium. The result is of some interest in showing that in the normal animal the anterior pituitary substance does not merely expedite the life history of the follicle, but actually causes a new phase of development in the follicle cell. It seems clear, therefore, that the luteinising hormone must be associated strictly with the post-ovulative phase of the cycle.

Contrary to what might have been anticipated, the effect on the X-rayed ovary does not seem to be irreversible. In 5 out of the 12 animals recorded in Table III œstrus reappeared within 11 days of the last injection. In only two others was time allowed before autopsy for the cycle to recommence. The histological evidence also shows that the luteinisation is not permanent. APX36, killed 12 days after the last injection and 4 days after the recommencement of œstrus, showed no trace of the characteristic effect (Plate 3, fig. 3), while in APX37, killed 13 days after the last injection and 2 days after the recommencement of œstrus, showed only areas of shrunken and degenerate luteal tissue (Plate 3, fig. 2).

The luteinisation of any follicles remaining in incompletely sterilised animals may have produced luteal tissue, but for the following reasons it is evident that such a factor can have played no significant rôle in the results described :—

- (a) The irregular and ill-defined nature of the patches of luteal tissue.
- (b) The absence of degenerate oocytes among the luteal tissue.
- (c) The absence of primordial follicles such as are found in the incompletely sterilised animal, and which would survive the effects of the extracts.

Injection of Anterior Pituitary Tissue Suspensions into Sterile Mice.—The ovulation-promoting preparation of the anterior pituitary body was injected into six irradiated mice. Neither histological effects on the ovary nor physiological effects on the cycle were observed from this administration to sterile animals (Plate 3, fig. 4).

I am greatly indebted to Prof. J. P. Hill, F.R.S., for facilities for working in the Department of Histology and Embryology, University College, while to Prof. G. Elliot Smith, F.R.S., my thanks are due for allowing the X-irradiations to be carried out in the Department of Anatomy.

The expenses have been defrayed from grants from the Medical Research Council, to whom my thanks are due.

The anterior pituitary preparations were made by Mr. G. F. Marrian, to whom I am much indebted for this assistance.

VI.—*Summary.*

1. The effects on the occurrence of œstrus of the elimination and stimulation of the luteal tissue of the ovary have been studied.

2. The postponement of the next œstrus by sterile copulation is not found in certain untreated, but abnormal, mice which fail to ovulate at œstrus, and which fail, therefore, to produce corpora lutea.

3. Elimination of the corpora lutea by X-ray sterilisation has a similar result in some instances, but in others secondary changes in the irradiated ovary obscure the result.

4. It was previously shown that the ovary is responsible for the œstrus inhibition found in the mouse during lactation (Parkes and Bellerby). By means of a unilateral sterilisation technique it is now shown definitely that the corpus luteum is the actual site of this inhibition.

5. The luteal stimulating and indirectly œstrus-inhibiting effect of NaOH extracts of anterior pituitary is confirmed on the mouse.

6. In the sterilised mouse the NaOH extract, finding no organised follicles to act upon, luteinises most or all of the parenchymatous proliferation of the germinal epithelium, which forms the bulk of the irradiated ovary. This luteinisation results in the suppression of œstrus in the sterilised mouse. The process is, however, both histologically and physiologically reversible, and the cycle recommences after the end of injection.

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DESCRIPTION OF PLATES 2-4.

Guide Letters.

D.O., degenerate ovum; *F.R.*, follicle remains; *G.E.*, germinal epithelium; *F.E.L.*, follicle epithelium becoming luteinised; *L.C.*, luteal cell; *L.T.*, luteal tissue; *N.F.*, normal follicle; *N.L.T.*, normal luteal tissue; *T.F.P.*, tissue of first post-irradiation proliferation; *T.S.P.*, tubule of second post-irradiation proliferation; *T.E.* theca externa.

Plates prepared from Photomicrographs by Mr. F. J. Pittock.

PLATE 2.

FIG. 1.—Ovary of AP25, injected NaOH extract of anterior pituitary, showing large numbers of corpora lutea produced. $\times 26$.

FIG. 2.—Corpus luteum of AM1, showing degenerate ovum, indicative of non-ovulation. $\times 250$.

FIG. 3.—Corpus luteum of AP27, injected NaOH extract of anterior pituitary, showing degenerate ovum in luteal tissue. $\times 250$.

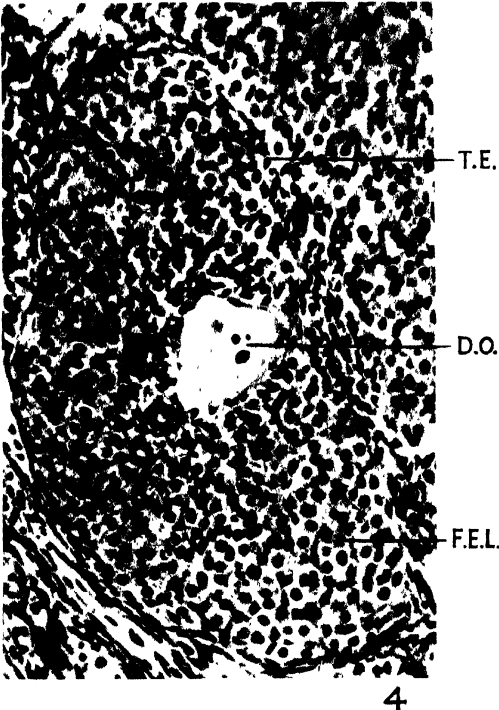
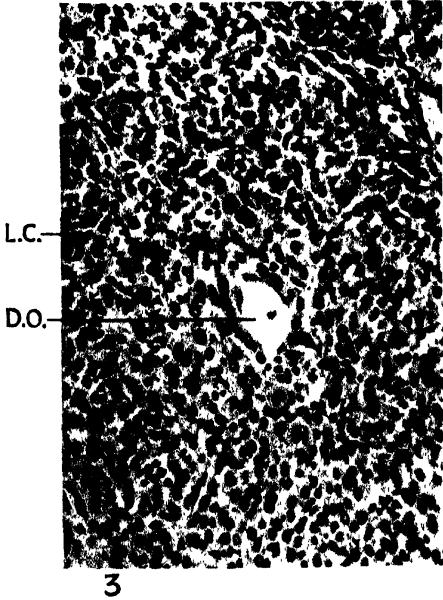
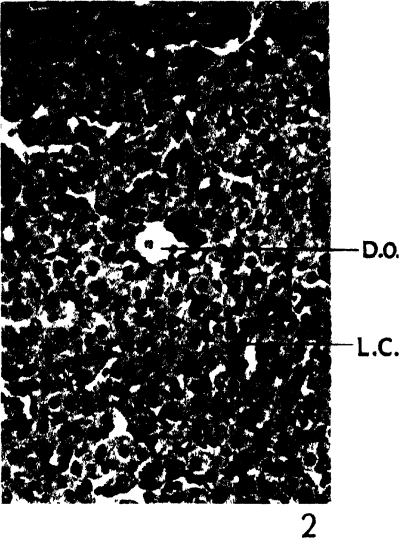
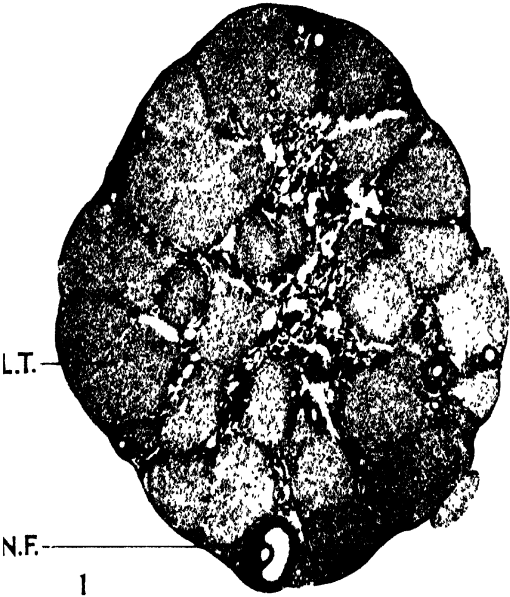
FIG. 4.—Follicle of AP32, showing process of conversion into corpus luteum under the influence of anterior pituitary extract. $\times 250$.

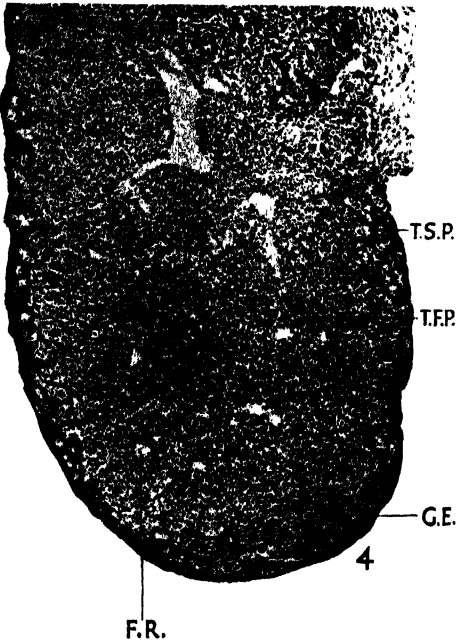
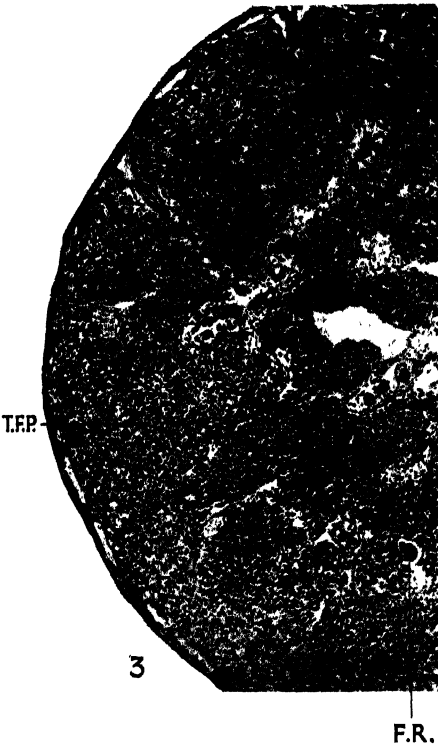
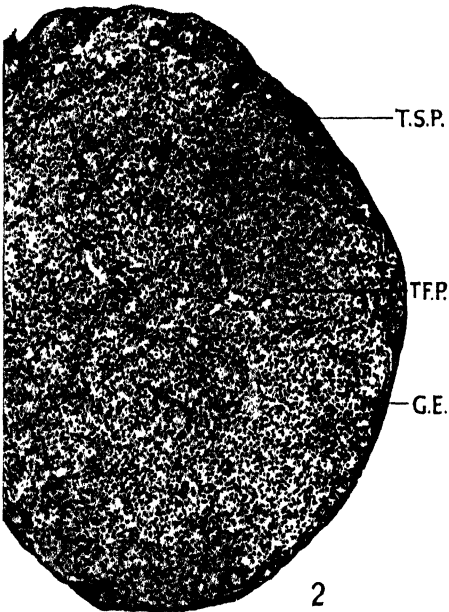
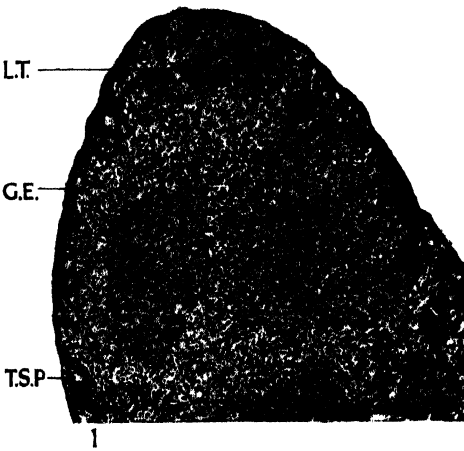
PLATE 3.

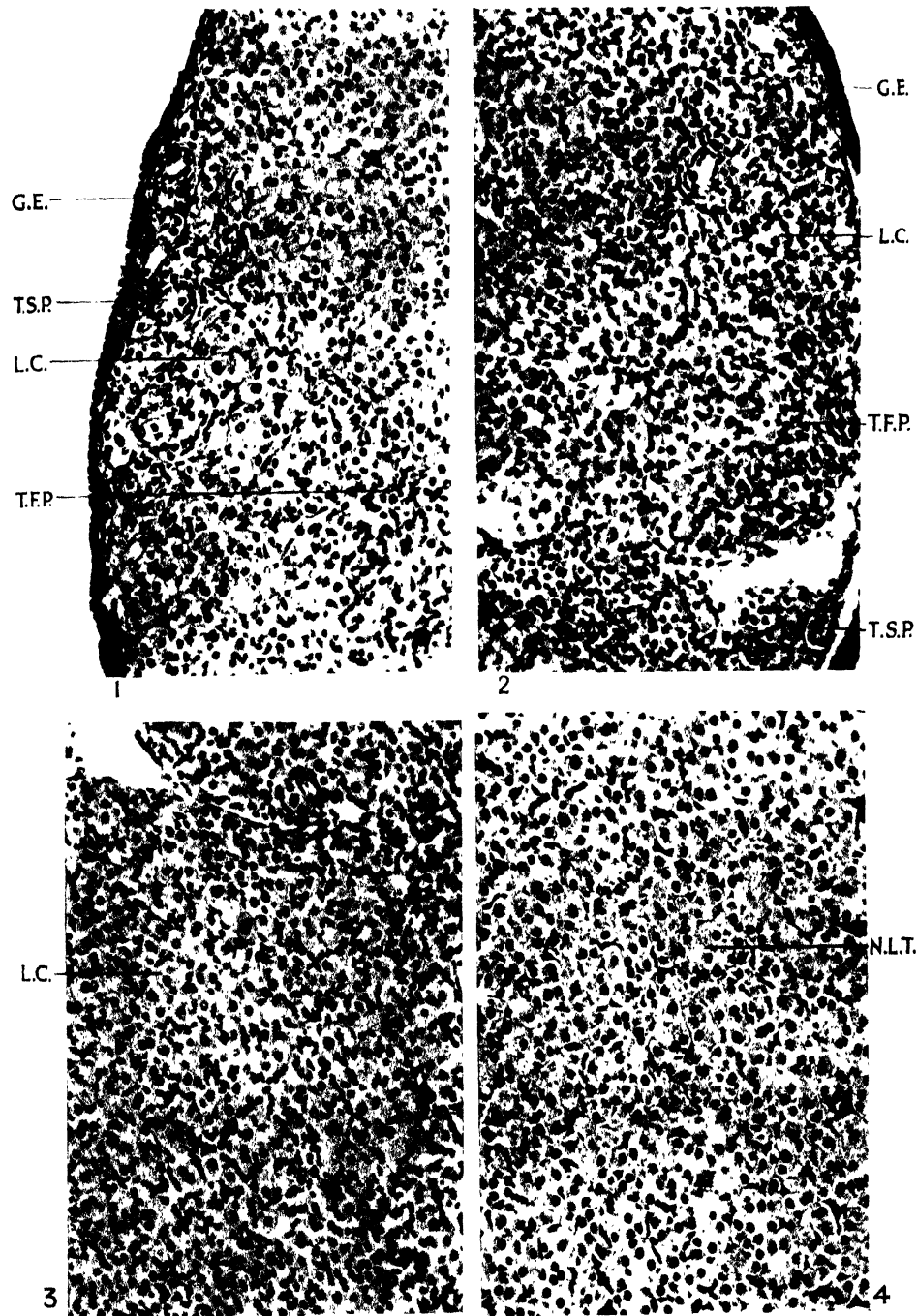
- FIG. 1.—Ovary of APX35, sterilised mouse injected with NaOH extracts of anterior pituitary, showing production of irregular patches of luteal cells in the first post-irradiation proliferation. This tissue should be distinguished from the old atretic corpus luteum on the right-hand side of the same section. (Estrus inhibited. $\times 75$.)
- FIG. 2.—Ovary of APX37, sterilised mouse 11 days after last injection of anterior pituitary extract. Estrus had reappeared. Showing lack of luteal-like cells as compared with APX35 (fig. 1). $\times 75$.
- FIG. 3.—Ovary of APX36, sterilised mouse 8 days after last injection of anterior pituitary extract. Estrus reappeared. Showing lack of luteal cells. $\times 75$.
- FIG. 4.—Ovary of APX40, showing non-effect of suspensions of mouse anterior pituitary tissue. Typical sterilised ovary. $\times 75$.

PLATE 4.

- FIG. 1.—Higher magnification of Plate 3, fig. 1 (APX35) showing luteal cells. Their diffuse arrangement shows that the luteal tissue has not been derived from an atretic corpus luteum. $\times 230$.
- FIG. 2.—Ovary of APX33, sterilised mouse injected NaOH extracts of anterior pituitary, showing diffuse luteal tissue. $\times 230$.
- FIG. 3.—Ovary of APX34, sterilised mouse injected NaOH extracts of anterior pituitary, showing diffuse luteal tissue. $\times 230$.¹
- FIG. 4.—Corpus luteum from normal pregnant mouse, showing similarity of luteal cells to those produced in the sterilised ovary by anterior pituitary extracts. $\times 230$.







The Functions of the Corpus Luteum. II.—The Experimental Production of Placentomata in the Mouse.

By A. S. PARKES (Beit Memorial Research Fellow).

(Communicated by C. L. EVANS, F.R.S.—Received October 9, 1928)

(From the Department of Physiology and Biochemistry, University College, London.)

[PLATE 5]

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I.—*Introduction.*

During the luteal phase of the cycle in many mammals, notably in the rabbit, ferret, and dog, the uterus undergoes changes which are designed to facilitate the implantation of the fertilised ovum. In other animals, such as the guinea-pig (5), the uterine changes during the luteal phase are histologically less obvious, but physiologically the uterine mucosa is in a peculiar condition of irritability. Injury to the mucosa at this time results in the production of large blocks of decidua-like tissue, to which the terms *placentomata* or *deciduomata* have been given. It has been shown by Marshall, Hammond, Loeb and others that the presence of the corpus luteum is essential for these post-ovulative uterine changes, and therefore that the corpus luteum is directly or indirectly responsible for their production.

In the rabbit decidual tissue can only be induced to develop when functional corpora lutea are present in the ovary. In the guinea-pig (Loeb, 5) *placentomata* can be produced during the post-ovulation phase of the cycle in the

unmated female, but in the rat Long and Evans (6) were unable to obtain a similar result. This discrepancy is undoubtedly due to the fact that in the short diœstrous cycle of the rat the corpora lutea undergo comparatively little development, whereas in the guinea-pig the cycle is longer and the corpora lutea are known to become active. During the pseudo-pregnancy which follows sterile copulation in the rat (Long and Evans, 6) and also during lactation (Corner and Warren, 2) placentomata can be produced. During both of these times corpora lutea develop to a greater extent than during the diœstrous cycle and become functional.

Since it is clear that luteal activity is required to produce the sensitivity necessary for placentomata formation, the occurrence of this sensitivity under experimental conditions of elimination or stimulation of the corpora lutea is of interest. Teel (8) has shown that the administration to the rat of NaOH extracts of anterior pituitary, which causes the wholesale production of luteal tissue by the follicles, makes possible the formation of placentomata during the ordinary diœstrous cycle. This has been confirmed by Brouha (1). More recently Weichert (9) has been able to sensitize the uterus by the injection of corpus luteum extract, (a) during the ordinary diœstrous cycle, and (b) after ovariectomy, provided œstrus had been induced first.

In view of these results it was decided to investigate the possibility of placenta formation in the mouse (a) during the phases of the normal sexual cycle; (b) after elimination of the corpora lutea; and (c) after stimulation of the luteal tissue.

II.—*Methods and Materials.*

The only technique employed, not described in previous papers, was the operation to induce placentomata formation. This was performed, as described by Long and Evans, by passing a thread transversely through the uterus, so that the lumen was penetrated and the uterine mucosa damaged.

III.—*Placenta Formation during the Normal Cycle.*

(a) *In Unmated Females.*—Five operations performed at various stages of the cycle in the unmated mouse failed to produce any signs of placentomata. Sensitization of the uterus does not therefore occur under these conditions.

(b) *During Pseudo-pregnancy.*—The operations performed during pseudo-pregnancy gave very definite positive results (Table I).

Table I.—Production of Placentomata during Pseudo-Pregnancy.

No. of mouse.	Time of operation (days post coitum).	Time of autopsy (days after operation)	Result.
SP1	5	3	Slight enlargement.
SP2	4	4	+
SP3	5	4	Negative (on œstrus).
SP4	3	4	+
SP5	5	4	Slight enlargement.
SP6	3	4	+
SP7	4	4	+
SP8	3	4	+
SP9	6	4	+
SP10	3	4	+
SP11	3	5	+

In SP3 the pseudo-pregnancy was abnormal, and the return of œstrus premature. This negative result must therefore be excluded. From the other animals the following conclusions may be drawn:—

- As in the rat, placentomata may be readily produced during pseudo-pregnancy.
- The maximum sensitivity of the uterus occurs about 3 days post coitum. Sensitivity is almost lost by 5 days post coitum.

The time of maximum reaction is thus somewhat earlier than that given by Long and Evans (6) for the rat.

A cross-section of the placentoma of SP6 is shown in Plate 5, fig. 2.

(c) *During Lactation.*—Placentoma formation occurred during lactation, Table II, but not to such an extent as was found during pseudo-pregnancy.

Table II. - Placentoma Formation during Lactation.

No. of mouse.	Time of operation (days post partum).	Time of autopsy (days after operation).	Result.
LC1	6	4	+
LC4	1	4	Negative.
LC5	5	4	+
LC6	7	4	+
LC7	4	4	+
LC8	4	4	+
LC9	5	4	+

The time of greatest reaction during lactation thus appears to be rather later than during pseudo-pregnancy, even allowing for the fact that the corpora lutea of lactation are not formed till some 24 hours after parturition.

This sensitivity of the uterus during lactation is of interest in again demonstrating the activity of the corpora lutea of lactation in the mouse, and in comparison with other mammals. In the rabbit oestrus is inhibited during lactation for what appear to be purely metabolic reasons, and the uterus, far from assuming a condition of pseudo-pregnancy, undergoes very obvious atrophy (Hammond, 4).

IV.—*Placentoma Formation under Experimental Conditions.*

Table III.—Placentoma Formation after Injection of Anterior Pituitary Extracts.

No. of animal.	Time of operation (days after beginning of injections).	Time of autopsy (days after operation).	Result.
UPAP7	4	4	
UPAP9	4	3	
UPAP13	4	1	Negative.
UPAP14	4	1	
UPAP15	4	4	+
UPAP16	5	4	++
UPAP17	5	1	Negative.

(a) *In Unmated Females receiving Anterior Pituitary Extracts.*—The results given in Table III show that placentomata were produced during the dioestrous cycle when NaOH extracts of anterior pituitary were administered. This finding confirms the results of Teel (8) and Brouha (1). The degree of development obtained was, however, markedly inferior (Plate 5, fig. 1) to that found during pseudo-pregnancy.

(b) *In Unmated X-irradiated Females.*—Since placentomata cannot be formed during the normal dioestrous cycle, it was not to be expected that they could be in the unmated X-irradiated animal deprived of all cyclic ovarian structures. Completely negative results were in fact obtained in a series of 13 mice.

(c) *After Sterile Copulation in X-irradiated Females.*—In Part I of this series (7) it was shown that in the absence of corpora lutea pseudo-pregnancy does not occur. It would be expected, therefore, that after obliteration of the corpora lutea by X-irradiation, the sensitivity of the uterus would not be found even after sterile copulation. Experiments showed this to be the case (Table IV).

In two only of these animals were placentomata produced, although all the operations were performed at what would have been the time of maximum

Table IV.—Attempts to Produce Placentomata after Sterile Copulation in X-irradiated Females.

No. of animal.	Time of operation (days post coitum).	Time of autopsy (days after operation).	Results	State of ovaries.
XSP1	4	4	Negative	Sterile.
XSP2	3	4	"	"
XSP3	3	4	+ + +	(foetus) 2 new corpora, 1 follicle.
XSP4	3	4	Negative	Sterile.
XSP5	3	2	"	"
XSP6	4	4	"	"
XSP7	3	4	"	"
XSP8	4	4	"	"
XSP9	3	4	"	"
XSP10	3	4	"	"

sensitivity. Of the two animals in question, one was unsterilised, two new corpora lutea and an embryo being present. This animal is of importance in showing that placentomata may be produced alongside actual embryos. The placentoma in this case was much larger than the whole conceptus, although only half the age, and it would be of interest to ascertain to what degree of development a placentoma would attain during pregnancy. XSP10, the other animal of this series to form a placentoma, was found to be completely sterile, and is thus an anomaly compared with the rest of the series.

(d) *In Unmated X-irradiated Females receiving Anterior Pituitary Extracts.*—In the previous paper it was shown that NaOH extracts of anterior pituitary will regularly cause complete luteinisation of the irradiated ovary, and bring about inhibition of œstrous. It was confidently expected that the sterilised ovary thus transformed would sensitize the uterus and make possible the formation of placentomata. In practice the results were disappointing and only negative results were obtained in spite of the very marked ovarian changes found (Plate 5, figs. 3 and 4). This is probably to be explained on the grounds that the abnormal conditions may have altered the time relations of the appearance of sensitivity.

V.—Summary.

I.—It was found possible to produce placentomata in the mouse :—

(a) During pseudo-pregnancy.

(b) During lactation.

- (c) In the dioestrous cycle after injection of NaOH extracts of anterior pituitary.

II.—Attempts to produce placentomata failed :—

- (a) During the normal dioestrous cycle in the unmated female.
 (b) In the unmated X-irradiated female.
 (c) In the X-irradiated female after sterile copulation (with one exception).
 (d) In the X-irradiated female after injection of anterior pituitary extracts.

The expenses of the work described above were defrayed from grants from the Medical Research Council, to whom my thanks are due.

I am greatly indebted to Prof. J. P. Hill, F.R.S., for allowing facilities for histological work, and to Prof. G. Elliot Smith, F.R.S., for allowing the irradiations to be carried out in the Department of Anatomy.

The anterior pituitary preparations were made by Mr. G. F. Marrian, to whom my best thanks are due for this assistance.

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DESCRIPTION OF PLATE 5.

Guide Letters.

L., lumen of uterus ; *S.*, stroma ; *M.L.*, muscle layer ; *P.T.*, placentoma tissue ; *L.C.*, luteal cell ; *F.R.*, follicle remains *T.F.P.*, tissue of the first post-irradiation proliferation (usual type).

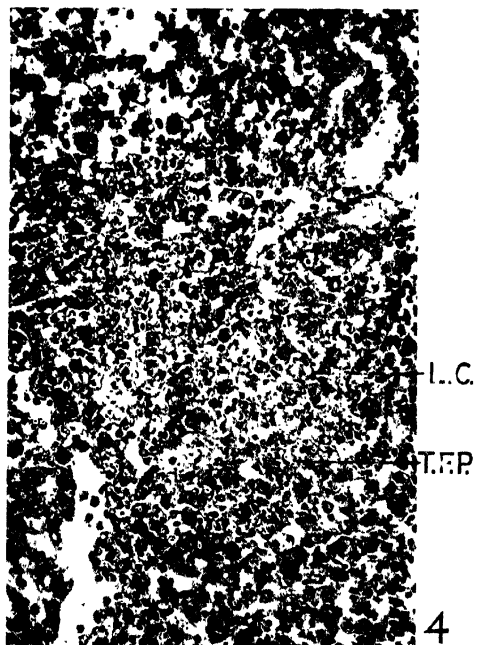
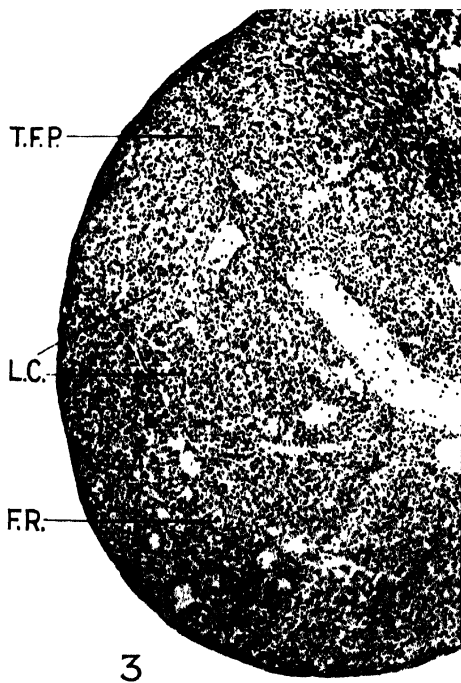
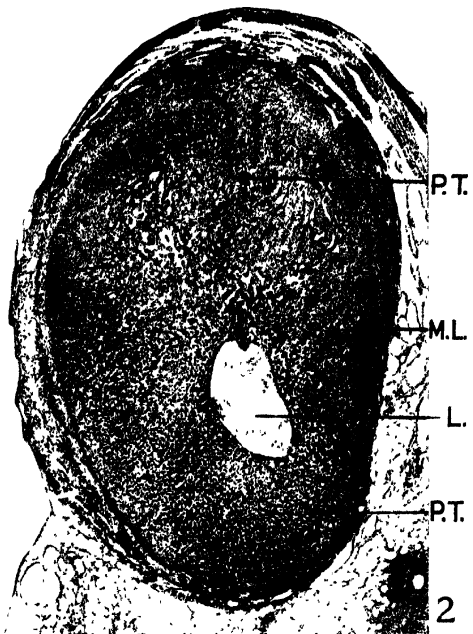
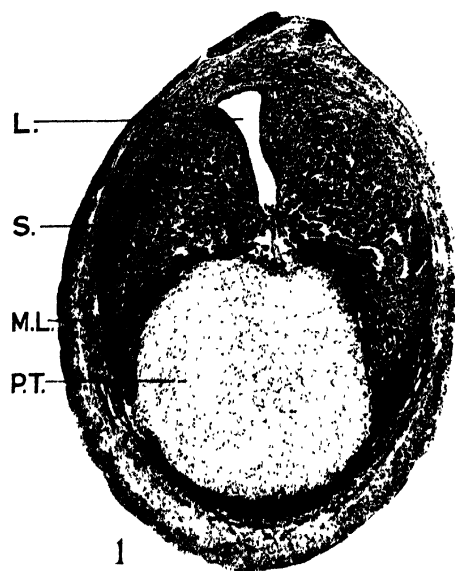
FIG. 1.—Section through the end of placentoma of UPAP7, unmated mouse injected with NaOH extract of anterior pituitary. $\times 23$.

FIG. 2.—Placentoma of SP6, produced during pseudo-pregnancy after sterile copulation. $\times 18$.

FIG. 3.—Ovary of XPAP3, showing lutealisation of the first post-irradiation proliferation. $\times 85$.

FIG. 4.—Higher magnification of portion of fig. 3, showing luteal cells interspersed in normal tissue of the post-irradiation proliferation. $\times 230$.

Photomicrographs by Mr. F. J. Pittock.



The Functions of the Corpus Luteum. III.—The Factors concerned in the Development of the Mammary Gland.

By A. S. PARKES (Beit Memorial Research Fellow).

(Communicated by C. L. EVANS, F.R.S.—Received October 9, 1928.)

(From the Department of Physiology and Biochemistry, University College, London.)

[PLATES 6–10.]

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I. *Introduction.*

Phases of Mammary Growth.—The development of the mammary gland from before puberty until the later stages of pregnancy may roughly be divided into four phases :—

- (a) During the pre-pubertal phase the mammary gland undergoes gradual development, though the gland is still limited to a few ducts in the neighbourhood of the nipple.
- (b) At puberty (*i.e.*, the first œstrus) growth in the ducts takes place. This phase is well marked in the guinea-pig (Loeb, 17), in the rabbit (Ancel and Bouin, 5), and in the opossum (Hartman, 14). In the absence of pregnancy this occurs at each œstrus.
- (c) These phases of growth, however, still leave the gland in a rudimentary condition, and after ovulation, correlated with the development of the corpus luteum, the third phase of growth of the mammary gland begins. If pregnancy fails to occur the subsequent removal of the luteal influence results in cessation of mammary growth after a greater or lesser development. The growth of the gland during the luteal phase of the cycle may begin rapidly (as in the rabbit) or there may be a hiatus between the development of œstrus and that of the luteal phase (as in the guinea-pig). Even during the luteal phase of the short diœstrous cycle in

the non-pregnant animal the gland may be built up sufficiently to admit of a certain amount of secretion (Hammond and Woodman, 13). Where the luteal phase is very pronounced, as in animals such as the rabbit, ferret and dog, the end of the very considerable growth phase is regularly accompanied by the secretion of milk (Ancel and Bouin, 3, 4; Hammond, 12; Marshall and Halnan, 18).

- (d) During pregnancy the ultimate growth of the mammary gland occurs and this far exceeds the development at any other time. Two phases of growth are found. In the early stages of pregnancy the mammary gland undergoes a degree of development comparable with that found during the luteal phase of the ordinary cycle or during pseudo-pregnancy. In the later stages, however, an entirely new phase of growth begins, which results in greatly increased size of the gland and which culminates in the full development of the gland as found at the end of pregnancy.

Nature of the Stimulus required for Mammary Growth.—The growth which takes place both before and at puberty can readily be shown by means of ovariectomy and grafting experiments to be dependent upon the ovary; since corpora lutea are not present up to this time the stimulus may be said to be ovarian, but other than luteal. The proliferation of the gland which occurs at œstrus may reasonably be supposed to be dependent upon the œstrus-producing hormone, and it seems clear that this hormone does bring about growth of the gland. [Herrmann (15), Fellner (8), Ancel and Bouin (5), Aschner (6), Frank and Rosenbloom (9), Allen (1), Hartman (14).] Much of this work has been designed to show that the œstrus producing hormone will cause complete development of the gland as found at the end of pregnancy. [Allen (1).]

So far, however, no adequate demonstration that œstrin will bring about full growth of the mammary glands has been forthcoming. Ancel and Bouin (5) and Vitemberger (19) definitely distinguish in the rabbit between the slight mammary growth characteristic of œstrus and the extensive hypertrophy characteristic of pregnancy and pseudo-pregnancy. The latter growth is not found in even the most prolonged œstrus and cannot be caused by injection of œstrin.

The growth which takes place during the luteal phase of the cycle and during pseudo-pregnancy, when the corpus luteum dominates the ovary, would be supposed to be dependent upon the corpus luteum, and this can be clearly demonstrated in such animals as the rabbit, dog and the ferret (Ancel and Bouin, 2, 3; Marshall, 18; Hammond, 12).

The nature of the stimulus required for the final development in the later stages of pregnancy is, however, more open to dispute. The rabbit and the dog provide no answer to this question, because the failure of pseudo-pregnant development to equal that of true pregnancy may be due either to the shorter duration of pseudo-pregnancy or to the absence of foetuses.

Experiments on the rabbit designed to throw light on this question have given no definite results. It is not possible, of course, to investigate the problem directly, by removing the corpora lutea during pregnancy: the operation results in abortion. Lane-Clayton and Starling (16) reported the induction of mammary growth with foetal extracts, but the greatest degree of growth was brought about by the use of placental extracts (which may have contained oestrin); in any case their illustrations make it evident that the degree of growth induced was far less than is found during ordinary pseudo-pregnancy, and it is known that the ovary alone is responsible for the growth at this time.

Hammond (11) endeavoured to throw light on the part, if any, played by the products of conception by (a) causing the formation of placentomata during pseudo-pregnancy, and (b) removing the foetuses during pregnancy but leaving the placentae. The results showed that placental tissue was not responsible for the final development of the glands.

Ansel and Bouin (2) attribute the final growth to a "uterine gland" - the myometrial gland, which according to these authors develops during pregnancy. This gland, however, appears to be very erratic in occurrence (Hammond, 12).

The most hopeful line of attack would clearly be to prolong pseudo-pregnancy by some means to the length of true pregnancy, and the means have now become available. During recent years much attention has been directed to the influence of various extracts of the anterior pituitary body upon the ovary, and as originally shown by Evans (7) injections of sodium hydroxide extracts of anterior pituitary into the normal animal result in great stimulation of the luteal tissue and in the induction of a prolonged luteal phase.

Two types of experiment, therefore, seemed possible on the rabbit:—

- (a) To induce pseudo-pregnancy without sterile copulation by injection of anterior pituitary extract, and to prolong this induced phase if possible.
- (b) To start the injection towards the end of normal pseudo-pregnancy and thus to endeavour to prolong the normal luteal phase.

In practice it was found that the extracts tended to cause local reactions in the rabbit, and the second type of experiment was therefore more successful.

As a result of prolonging normal pseudo-pregnancy, complete development of the mammary gland as found at the end of normal pregnancy has been produced. It may therefore be said with confidence that no foetal factor is required for the complete development of mammary tissue.

II. *Methods and Materials.*

Animals used for the induction of pseudo-pregnancy were kept one in a cage for at least a month, and then a laparotomy was performed, in order to make certain that no corpora lutea were already present in the ovary. This was a necessary precaution because the animals were of unknown history, and in any case rabbits will occasionally ovulate spontaneously.

Ordinary pseudo-pregnancy was induced by mating with a vasectomised buck and the injections of anterior pituitary extract were begun at about 12 to 14 days pseudo-pregnant. The extracts were made from the directions given by Evans and by Teel, described in Part I of this series. 5.0 c.c. per day was given. The mammary gland preparations were made by the method described by Hammond, which consists of stripping the gland away entirely from the skin, fixing, staining and mounting entire in Canada balsam. The photographs shown in this paper were made from such preparations, and are not retouched in any way. Material for histological examination was fixed in Bouin's fluid and stained in Ehrlich's hæmatoxylin and eosin. Sections were cut at 7 μ .

III. *The Development in the Normal Rabbit.*

The development of the mammary gland in the normal rabbit has been very fully dealt with by several workers, notably by Hammond, and only the barest outline need be given here. In the pre-pubertal animal the gland is limited to a few small ducts in the neighbourhood of the nipple (Plate 7, fig. 1). At the time of the first œstrus the ducts grow out radially from the nipple, but still remain very thin. The maximum of this stage of growth, possibly superimposed on the vestiges of previous pseudo-pregnant growth, is shown in Plate 7, fig. 2. So long as the animal remains on œstrus the development remains static at this point. During pseudo-pregnancy the ducts branch and thicken greatly and the gland takes on a typical fern-like appearance (Plate 7, fig. 3). The maximum development of this phase is reached at about 14 days *post-coitum*. Retrogression of the gland then takes place, leading to decrease in size and appearance of milk in the ducts (Plate 7, fig. 4).

During the second half of the pregnancy, instead of retrogression of the growth as found at the end of pseudo-pregnancy, intense growth sets in, and

thickening of the gland takes place. Plate 7, fig. 5 shows the mammary tissue at 23 days pregnant, while the complete growth (29 days pregnant, when the whole gland weighs 100-120 gms.) is shown in Plate 7, fig. 6.

Pseudo-pregnant development of the uterus is characterised by increase in size and by great proliferation of the uterine mucosa (Plate 10, fig. 3).

IV. Induced Pseudo-pregnancy.

Six rabbits known to have no corpora lutea in the ovaries were injected with the anterior pituitary extracts. Of these one died in 6 days, and another became ill. The rest appeared to suffer no adverse general effects from the injections, which on the whole, were absorbed fairly well.

The first four animals were injected for 15, 11, 18 and 18 days, autopsy being performed as soon as the injections were stopped. The first dissection showed that the effects of the anterior pituitary extracts on the ovaries were identical with those described on rats and mice. The ovaries were very large and full of corpora lutea, which were wedged together so closely as to be uncountable. Probably, however, between 30-40 were present in the two ovaries. Plate 6, fig. 2, shows a part of a typical section of one ovary of the animal injected for 15 days (MGR4). A number of blood follicles were present. As in rats and mice after similar treatment, the corpora lutea had been formed without ovulation, many in fact being embedded deep in the ovary. A large proportion were hollow owing to the retention of liquor folliculi, around which the follicular epithelium had been luteinised. The luteal tissue, however, appeared remarkably healthy (Plate 6, fig. 3).

In correlation with this production of luteal tissue in the ovary, growth of the mammary gland at least equal to that found during ordinary pseudo-pregnancy had occurred in every case. Plate 8, fig. 1, shows one nipple area of rabbit MGR4. In one part of this gland the secondary thickening typical of true pregnancy actually appears to have started. In an endeavour to prolong this induced pseudo-pregnancy and to bring about complete development of the mammary gland, the next rabbit was injected for 25 days, but during the last week the animal became ill and no satisfactory result was obtained.

The uterus during induced pseudo-pregnancy undergoes precisely the same changes as after normal ovulation (Plate 6, fig. 4). Since it seemed advisable to curtail the period of injection as far as possible, it was decided to begin injection after sterile copulation, and thus to prolong ordinary pseudo-pregnancy.

Control Experiments.—Control injections into two males and two ovarioto-

misled females gave no results whatever. It is thus clear that the anterior pituitary substance functions entirely through its effect on the ovary, and almost certainly through the luteal tissue produced.

V. *Prolonged Pseudo-pregnancy.*

The histories of the five animals in which injections were begun towards the end of ordinary pseudo-pregnancy are summarized in Table 1.

Table 1.

No. of rabbit.	Days pseudo-pregnant when injection started.	Days injected.	Time of killing after last injection (days).	Weight of gland (gm.).
MGR6	13	15	1	56
MGR10	14	13	1	40
MGR11	14	18	5	33
MGR12	14	10	0	38
MGR16	13	13	0	95

In at least one of these animals (MGR16) growth had proceeded to the full extent found at the end of normal pregnancy. A photograph of one complete nipple area is shown in Plate 8, fig. 4, while a section of the gland is shown in Plate 9, fig. 5. Three other animals showed stages in between complete pseudo-pregnant and complete pregnant growth (Plate 8, figs. 2 and 3).

One animal (MGR11) was left for 5 days after the last injection and at autopsy, though the gland itself had undergone atrophy, very large quantities of milk were found in the ducts. It is probable that this animal would have reared young, but unfortunately opportunity was not found to attempt this.

Two possible criticisms may be urged against these results: (a) the irregularity of the degree of growth produced the rabbit injected for 15 days showed less growth than that treated for 13 days, for instance; (b) the macroscopic and microscopic coarseness of the mammary tissue of the experimental animals as compared with the normal controls. (See the illustrations.) However, the probability that the growth-promoting stimulus produced under the experimental conditions may have been very irregular in its action, offers an adequate explanation of both of these criticisms.

The experiments seem to show quite definitely that if luteal action is prolonged, complete growth of the mammary gland will take place, and therefore that the prolonged life of the corpus luteum during pregnancy is sufficient to

bring about the final phase of mammary growth without any foetal action being required.

Finally, it may be pointed out that these results (especially those on MGR11) show fairly definitely that no foetal or other hormone is required to inhibit the secretion of milk during pregnancy, as has sometimes been supposed (10). The breakdown changes in the gland which occur when the building-up luteal stimulus is withdrawn appear to initiate secretion.

VI. *Summary.*

I. In the rabbit, the phase of mammary growth which takes place during the first half of pregnancy is also found during pseudo-pregnancy and cannot be dependent upon any foetal stimulus. It has been shown quite definitely (Ancel and Bouin, Hammond) that the corpus luteum is responsible for this phase.

II. The failure of mammary growth as found during the second half of pregnancy to occur in pseudo-pregnancy might be due to either (*a*) the shorter duration of pseudo-pregnancy, or (*b*) the absence of a foetal stimulus during pseudo-pregnancy.

III. Luteal stimulating extracts of anterior pituitary (Evans, 7) were used to prolong the luteal phase in the non-pregnant rabbit (pseudo-pregnancy) to the length of true pregnancy. As a result of this the final phase of mammary growth occurred in the absence of fetuses.

IV. No foetal factor is therefore required for the complete development of the mammary gland -only prolonged luteal action.

I should like to take this opportunity of expressing my gratitude to Mr. J. Hammond, School of Agriculture Cambridge, without whose advice and encouragement it would have been impossible to have carried out this work. All the normal rabbit material studied was given to me by Mr. Hammond.

To Prof. J. P. Hill, F.R.S., I am indebted for facilities for histological work. As before, the expenses were defrayed from grants from the Medical Research Council. My best thanks are due to Mr. G. F. Marrian for preparing the anterior pituitary extracts, and to Miss R. Deanesly for histological assistance.

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DESCRIPTION OF PLATES 6-10.

Guide Letters.

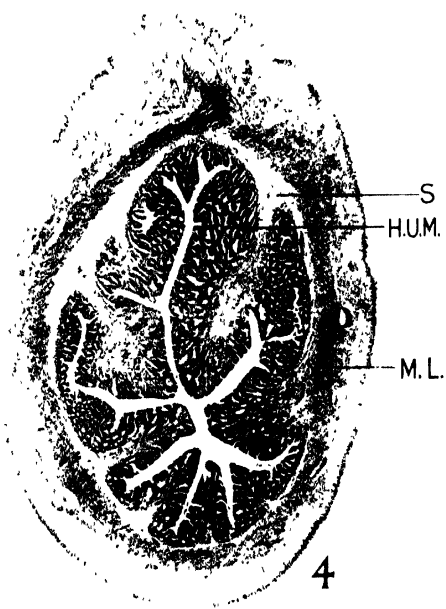
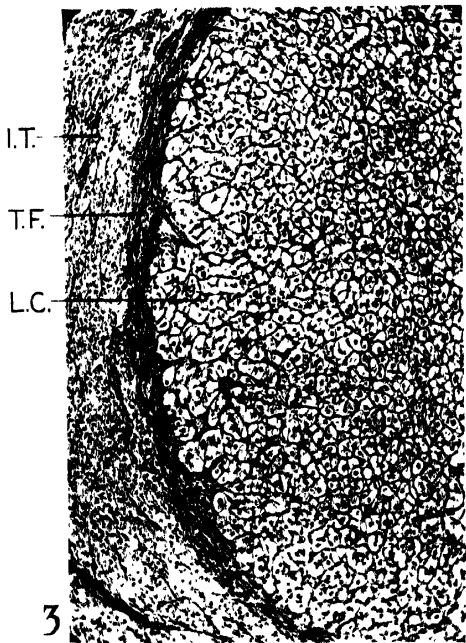
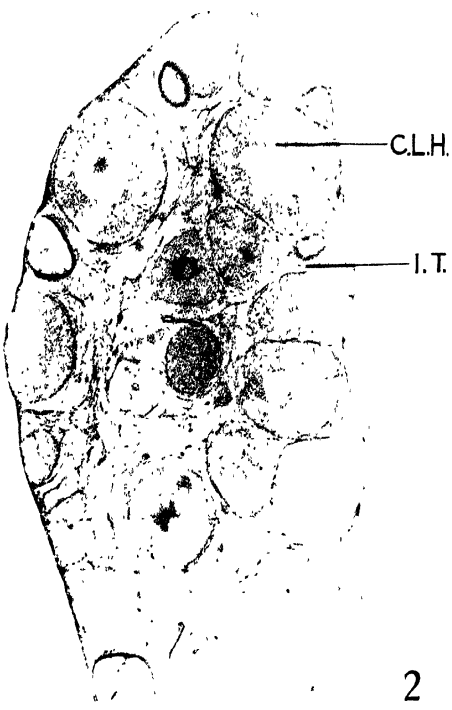
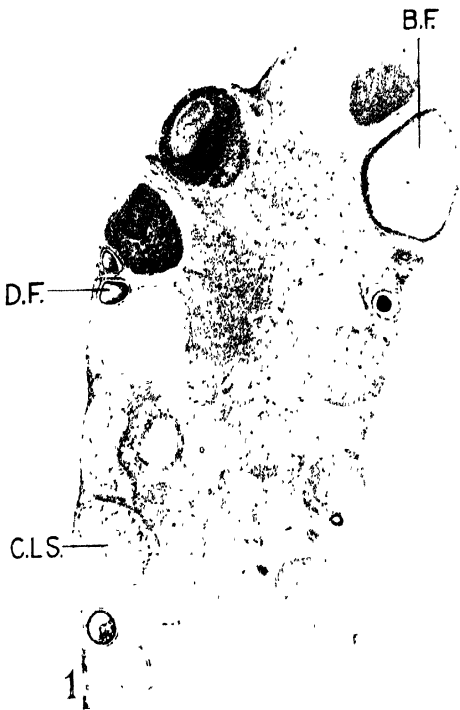
A., alveolus; A.P., alveolus during pregnancy; A.S., alveolar secretion; A.W., alveolar wall; B.F., blood follicle; C.L.H., hollow corpus luteum; C.L.S., solid corpus luteum; C.T., connective tissue between lobes of gland; D.F., degenerative follicle; G.N., gap between nipple areas; H.U.M., hypertrophied uterine mucosa; I.T., interstitial tissue; L.C., luteal cell; M.D., mammary duct containing milk; M.L., muscle layer; N., nipple; P.D., proliferating ducts; P.S., pseudo-pregnant growth of mammary gland; R.A., rudimentary alveolus; R.D., rudimentary ducts; S., stroma; S.T., secondary thickening of gland; T.E., theca externa.

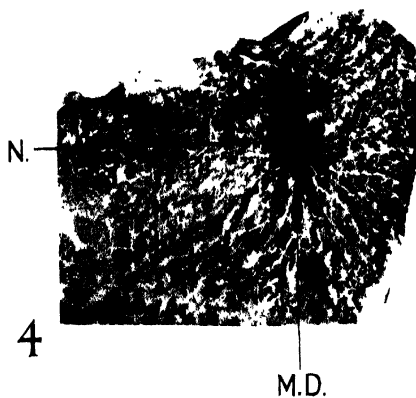
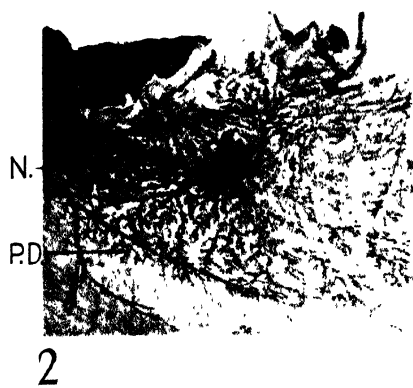
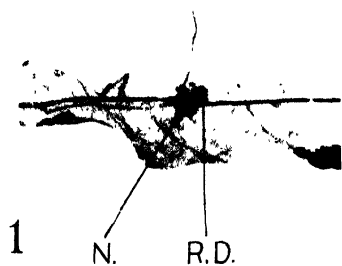
PLATE 6.

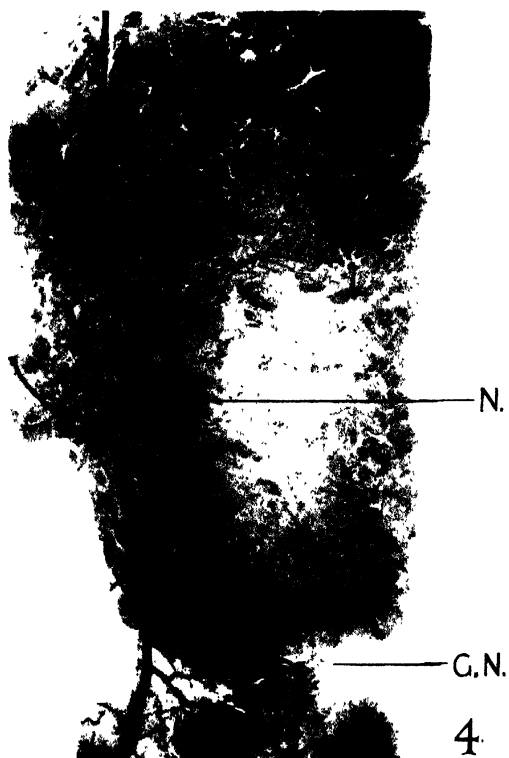
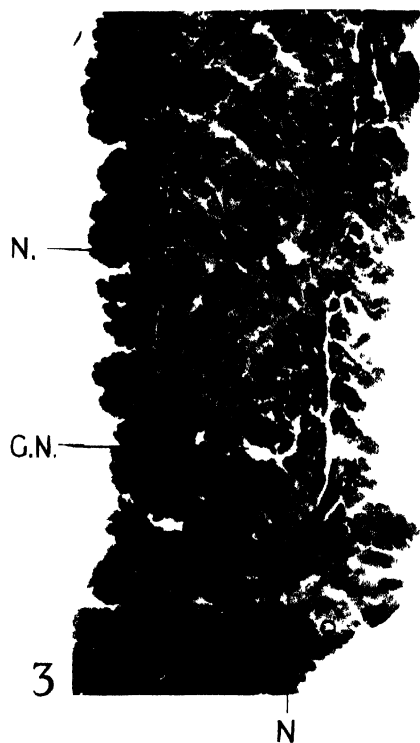
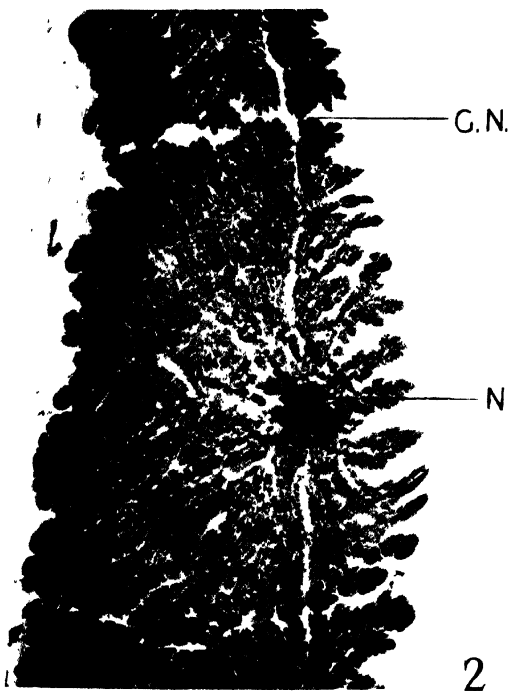
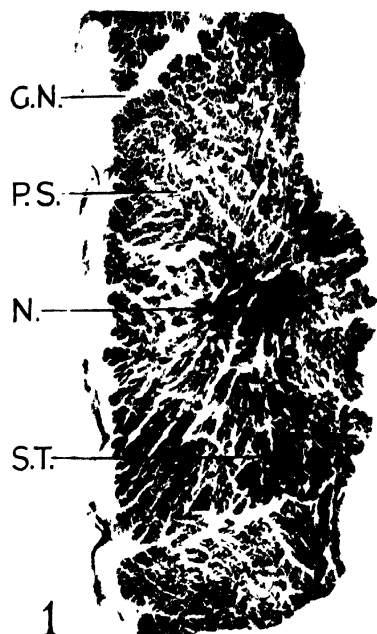
- FIG. 1. Ovary of MGR16, pseudo-pregnancy prolonged by injection of anterior pituitary extracts. $\times 8$.
- FIG. 2. Ovary of MGR4, pseudo-pregnancy induced by injection of anterior pituitary extracts without copulation. $\times 8$.
- FIG. 3.—Part of one of the corpora lutea of MGR4, showing the healthy nature of the luteal cells. $\times 70$.
- FIG. 4. Uterus of MGR4, showing typical pseudo-pregnant development. $\times 16$.

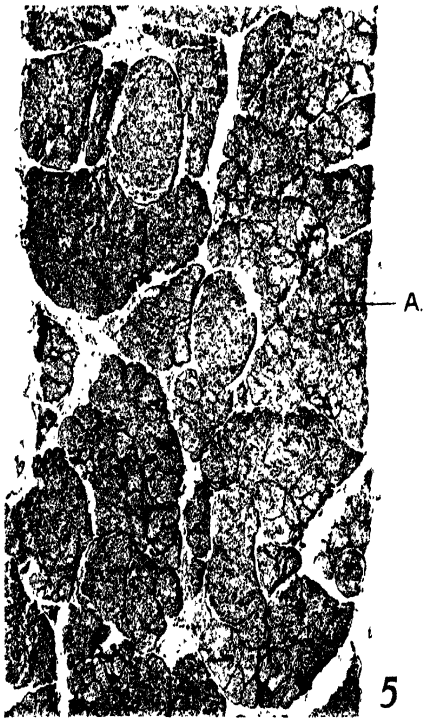
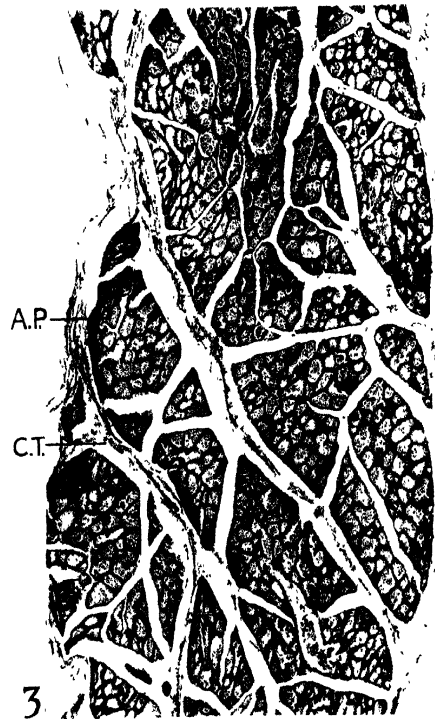
PLATE 7.

- FIG. 1.—Rudimentary mammary gland of pre-pubertal rabbit. $\times \frac{1}{4}$.
- FIG. 2.—Mammary gland of non-parous oestrous rabbit. This animal may have been pseudo-pregnant at an earlier date, and the growth shown probably represents oestrous development superimposed on the vestiges of a previous pseudo-pregnant growth. $\times \frac{1}{5}$.
- FIG. 3.—Mammary gland of a 12-day pseudo-pregnant rabbit. $\times \frac{1}{4}$.
- FIG. 4.—Twenty-first day after sterile copulation. Degeneration has set in, resulting in the presence of milk in the ducts. $\times \frac{1}{5}$.









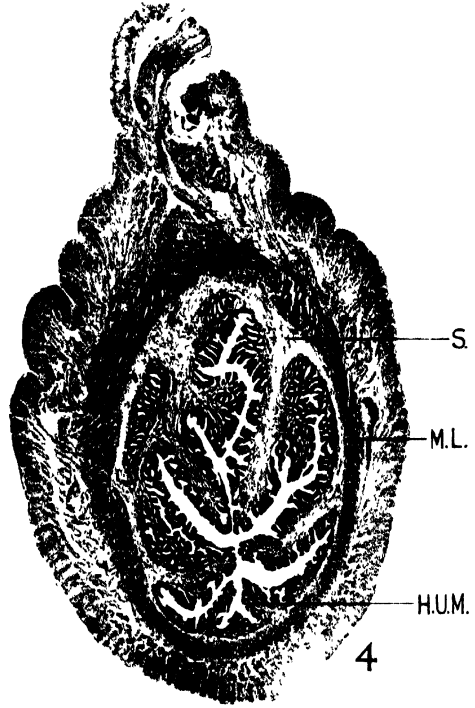
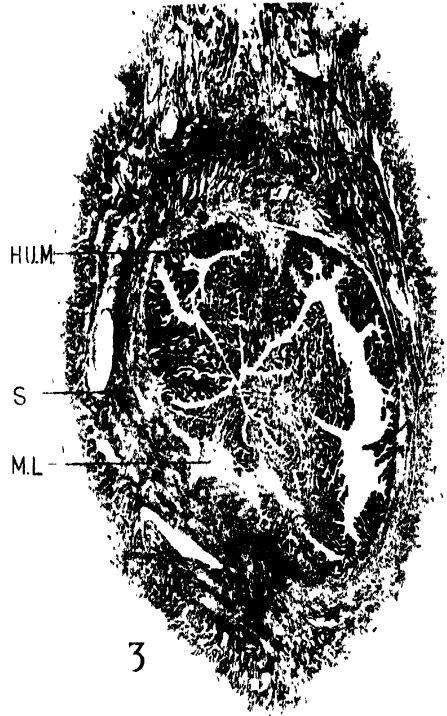
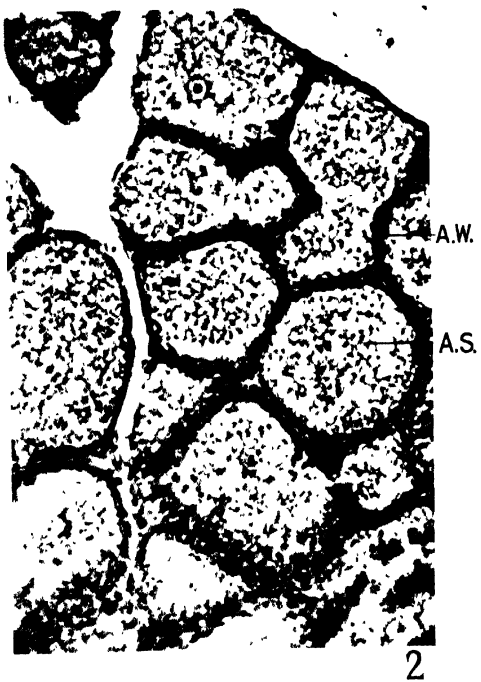


FIG. 5.—Twenty-third day of pregnancy, showing continuation of pseudo-pregnant growth. $\times \frac{1}{4}$

FIG. 6.—Twenty-ninth day of pregnancy. Only part of one nipple area is shown. The thickening of the gland as compared with pseudo-pregnancy is evident. $\times \frac{1}{5}$.

PLATE 8. (See Table I.)

FIG. 1.—Mammary gland of MGR4, showing induction of complete pseudo-pregnant growth and beginning in one corner of secondary thickening. $\times 1$.

FIG. 2.—Mammary gland of MGR10, showing secondary thickening well begun. The gaps between the nipple areas have not yet been entirely occluded. $\times 1$.

FIG. 3.—Mammary gland of MGR12, showing further stage of the secondary thickening. $\times 1$.

FIG. 4.—Gland of MGR16, showing induction of complete growth as found at the end of normal pregnancy. $\times 1$.

PLATE 9.

FIG. 1.—Section of mammary gland of 12-day pseudo-pregnant rabbit. (See Plate 7, fig. 3.) $\times 25$.

FIG. 2.—Section of gland of 23-day pregnant rabbit. (See Plate 2, fig. 5.) $\times 25$.

FIG. 3.—Section of gland of 29-day pregnant rabbit. (See Plate 2, fig. 6.) $\times 25$.

FIG. 4.—Section of gland of MGR12. (See Plate 3, fig. 2.) Showing similarity to 23-day pregnant stage. $\times 25$.

FIG. 5.—Section of gland of MGR16, showing similarity to 29-day pregnant stage, except in irregularity of size of alveoli and thinness of their walls. $\times 25$.

PLATE 10.

FIGS. 1 and 2.—Showing irregularity of alveoli and thinness of their walls in the gland of MGR16 (see Plate 9, fig. 5) as compared with normal 29-day pregnant stage. (See Plate 9, fig. 3.) $\times 215$.

FIG. 3.—Uterus of MGR5, showing typical pseudo-pregnant development. $\times 14$.

FIG. 4.—Uterus of MGR12 (prolonged pseudo-pregnancy) showing lack of development beyond that characteristic of ordinary pseudo-pregnancy. $\times 14$.

(Photographs and photomicrographs by Mr. F. J. Pittock.)

The Ultra-Violet Absorption Spectra of Certain Aromatic Amino-Acids, and of the Serum Proteins.

By FRANK CAMPBELL SMITH.

(Communicated by C. Lovatt Evans, F.R.S.—Received October 23, 1928.)

(From the Physiological Department, London Hospital Medical College.)

The absorption spectra of the aromatic amino-acids and of the serum proteins have been investigated by Dhéré, 1909 (1), who obtained values for the wave-lengths in close accordance with those found by subsequent workers; he was unable to measure the extinction coefficients, since at the time no suitable apparatus had been devised. He further noted that the absorption spectrum of tyrosine moved towards the red end of the spectrum when in an alkaline solution. He maintained that tyrosine and tryptophane were responsible for the absorption spectrum of protein.

In 1916, Kober (2) investigated the absorption bands of the aromatic amino-acids. Ward (3) in 1923, and Marchlewski (4) in 1925, made use of the rotating sector to measure the extinction coefficients of tyrosine, tryptophane, and phenyl-alanine. The absorption spectrum of tryptophane has also been measured by Abderhalden and Haas (5). In 1922, Judd Lewis (6) measured the absorption spectra of the serum proteins. Stenstrom and Reinhard (7) have confirmed the work of Dhéré, showing that the aromatic amino-acids present in the protein molecule were responsible for its absorption spectrum.

It appeared to the writer that, since the method of spectrophotometry is of great potential value in biological research, it would be worth while to repeat, and if possible to amplify and correlate the work of these observers. It is obvious that the samples of acids and of proteins employed should be of the highest possible standard of purity.

Method.—A Hilger quarter-plate spectrophotometer and rotating sector were used. The source of radiation was a condensed spark between tungsten steel electrodes.

Experimental.—The acids employed were obtained from the Hoffmann-La Roche Chemical Works, Ltd. The optical rotations of the *l*-tryptophane and *l*-tyrosine respectively were $[\alpha]_D^{20} = -32^\circ$ and $[\alpha]_D^{20} = -7.5^\circ$. In the case of tryptophane, the concentration was 0.5 per cent. in water; for tyrosine the concentration was 4 per cent. in a 21 per cent. solution of HCl in water

The nitrogen content of the *d-l*-phenylalanine was 8.68 per cent. Dilutions of a molecular solution in distilled water were employed in each case. The layer of fluid was 1 cm. in thickness. The height of the curve represents the molecular extinction coefficient (M). Ward in his paper (3) makes use of the following formula :—

$$M = \frac{\log I - \log I'}{C} \times \frac{1}{D},$$

where I = the intensity of the light entering the solution, I' = the intensity of the light leaving the solution, C = the molecular concentration and D = the thickness of the layer.

Curves were plotted on semi-logarithmic paper, the extinction coefficient being plotted on the logarithmic scale ; this was done in order to reduce the height of the curve to convenient dimensions.

The curves of tyrosine, tryptophane, and phenyl-alanine are shown in fig. 1.

Tyrosine. It will be seen that two new bands at wave-lengths of \AA 2240 and \AA 1940 have been observed in the case of tyrosine. It was found necessary to use Schumann plates to observe the band at the lower wave-length, since the absorption of gelatin obscured it. The band at the wave-length of \AA 2750 is in fairly close agreement with that found by Ward.

Tryptophane.—In the case of tryptophane two bands have been observed at wave lengths of 2790 and 2180 \AA , agreeing with those found by Hicks (8). The extinction coefficient at the foot of the curve as found by Ward is considerably higher than that found by the writer ; it is probable that in the former case the higher value was due to an impure sample of tryptophane.

Phenyl-alanine. Only one absorption band has been observed in the case of phenyl-alanine. No trace of the small bands described by Ward has been found. Since tryptophane and tyrosine, with a more complex structure than phenyl-alanine, give simple curves, one would not expect a curve with subsidiary maxima in the case of phenyl-alanine. The curve obtained by Ward is probably due to faulty technique or to an impure sample.

Serum Proteins.

The proteins employed were kindly prepared by Dr. L. F. Hewitt, who describes the method of preparation in his paper on the optical rotatory power of the serum proteins (9). The proteins thus obtained were of exceptional purity.

Six preparations of horse and six of human protein were used. The con-

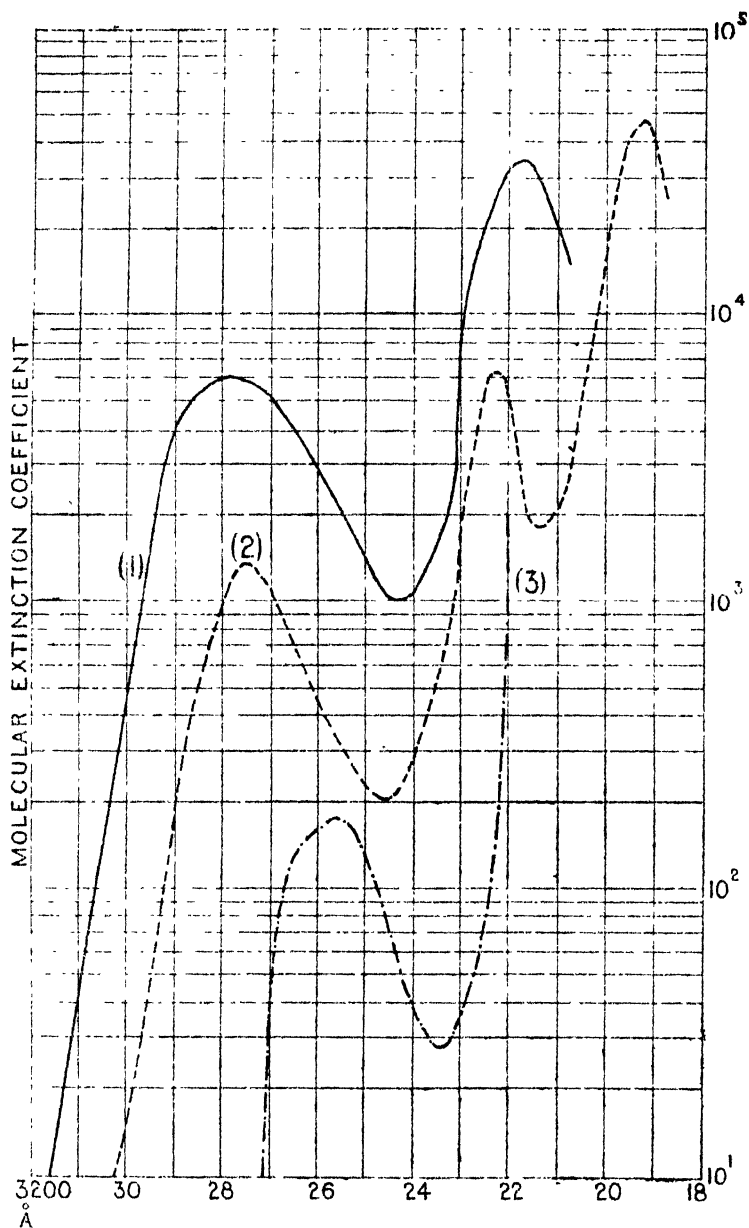


FIG. 1.

(1) Tryptophane. (2) Tyrosine. (3) Phenyl-alanine.

centration was measured by Kjeldahl's method, the original solution being about 6 per cent. This was then diluted to a concentration of 0.1 per cent.

and this concentration was employed in the absorption tube. The albumins were dissolved in distilled water, and the globulins in 0.1 per cent. saline, the control tube in each case containing the solvent. The layer of fluid was 1 cm. in thickness ; its absorption was measured at a P_H of 7.

Since it is possible that a protein solution, although apparently optically clear, might cause scattering at the wave-lengths at which its absorption was measured, and thus introduce an element of error, the following experiment was performed. A spectroscope was modified in such a way that a cell 1 cm. thick could be placed between the collimating lens and the prism. It is clear that, should the protein particles cause scattering, the lines of the spectrum at the corresponding wave-lengths would become ill-defined. In the case of the proteins employed no evidence of such scattering was observed.

Proteins, as it is well known, are precipitated by ultra-violet radiation ; it was therefore thought possible that exposure to the condensed spark in the process of photography might bring about some change in the protein and thus form a further source of error. The absorption of a sample of protein was measured, after which the same sample was exposed to the radiation from the condensed spark, the exposure being three times as long as that required for the photography of its absorption spectrum ; the absorption was then measured again and was found to be unaltered. Thus it is clear that the length of exposure used in these experiments can have no effect on the results obtained.

Further, in order to show that no diffusible substances were present, which might add to the absorption and thus form a source of error, a sample of each protein was ultra-filtered and the absorption of the filtrate measured. In all cases it was found to be diacetic.

The wave-lengths at head and foot of the curve in each case agreed within 10 Angström units ; the extinction coefficients at head and foot agreed within the limits of experimental error (4 per cent.). The average figures are shown below. Since the ratio $\frac{\text{extinction coefficient at head of curve}}{\text{extinction coefficient at foot of curve}}$ (or $\frac{\epsilon_h}{\epsilon_f}$) is an index of the purity of the sample, it is included in the table in each case.

Table I.

Proteins (concentration 0.1 per cent.).	Wave-length.		Extinction coefficient.		ϵ_h/ϵ_f .
	Head.	Foot.	Head ϵ_h .	Foot ϵ_f .	
Horse albumin	2790	2530	0.58	0.26	2.24
Human albumin	2790	2530	0.58	0.27	2.14
Horse pseudo-globulin	2790	2520	1.20	0.40	3.00
Human pseudo-globulin	2790	2520	1.20	0.40	3.00

For purposes of comparison, Judd Lewis' figures are given in Table II.

Table II.

Proteins (concentration 0.1 per cent.).	Wave-length.		Extinction coefficient.		ϵ_h/ϵ_f .
	Head.	Foot.	Head ϵ_h .	Foot ϵ_f .	
Horse albumin	2785	2545	1.20	0.85	1.4
Human albumin	2780	2540	0.675	0.45	1.5
Horse pseudo-globulin	2785	2520	1.20	0.475	2.55
Human pseudo-globulin	2780	2540	1.35	0.625	2.1

The curves for albumin and for globulin are found in fig. 2.

It will be seen in Table I that the values are identical for human and for horse protein. Judd Lewis, on the other hand (*v.* Table II), found that the extinction coefficient at the head of the curve of horse albumin was nearly double the value given for human albumin; he further found a difference in the ϵ_h/ϵ_f ratio in the two cases. He suggested that these results indicated either a physical difference in the state of aggregation of the two proteins, or possibly a chemical difference between horse and human protein.

In the present writer's opinion, this suggestion is unjustifiable, in the face of his own results; as is seen above, he is unable to confirm Judd Lewis's findings. He suggests that the discrepancy is due to the fact that his own samples of protein were of a higher standard of purity than those used by Judd Lewis.

The method of absorption spectrophotometry is undoubtedly of value in

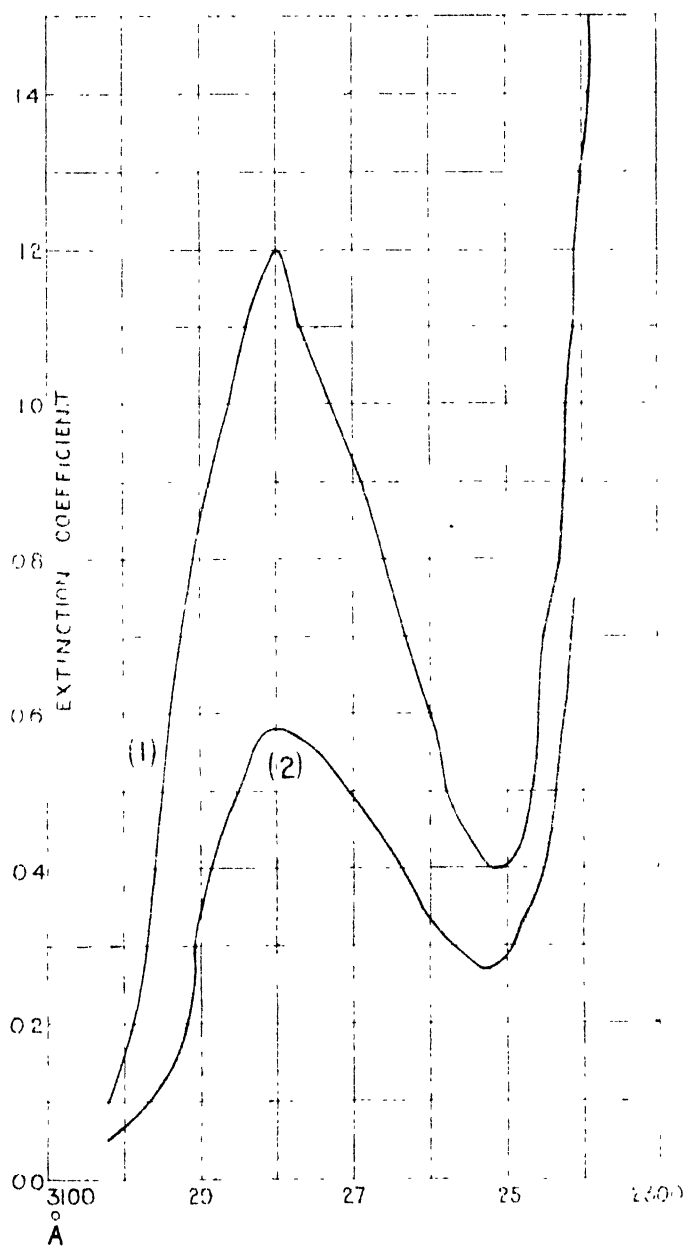


FIG. 2.

(1) Pseudo-globulin.

(2) Albumin.

measuring low concentrations of protein in small quantities. It has been successfully employed, for example, by Svedberg (10) in his work on the mole-

cular weight of egg albumin ; his extinction coefficients for this protein agree closely with those obtained by the present writer.

Further, it is seen above that the extinction coefficient for globulin at the head of the curve is nearly double that for albumin ; thus if the total amount of protein is known, the $\frac{\text{albumin}}{\text{globulin}} \left(\frac{A}{G} \right)$ ratio in a mixture of the two can be easily and accurately calculated from the following formula :—

$$\frac{A}{G} = \frac{E_h - 12x}{5.8x - E_h},$$

where E_h = extinction coefficient of mixture at head of absorption curve.
 x = total concentration of protein per cent.

If the total amount of protein is not known, the formula below may be used to ascertain both the ratio between the two proteins and the total concentration of protein. In order to avoid a large error in the final result, the extinction coefficients must be measured with great accuracy.

$$\frac{A}{G} = \frac{1.5E_f - 0.5 E_h}{0.325E_h - 0.725E_f},$$

where A/G = ratio of per cent. albumin to per cent. globulin, E_h = the extinction coefficient at the head of the curve given by the mixture, and E_f the extinction coefficient at the foot of the curve. The figures in the first formula represent the extinction coefficients of the standard curves of albumin and globulin, as shown in Table I.

The writer has found the application of the first formula useful in measuring the ratio of albumin to globulin in small amounts of cerebro-spinal fluid. If the spectrophotometric method is to be of real service, it is essential to have reliable figures available as a standard ; the writer, in undertaking this investigation, has endeavoured to establish curves which may be taken as reliable.

Summary.

1. The ultra-violet absorption spectra of tyrosine, tryptophane, and phenyl-alanine, and of the serum proteins have been measured.
2. Two new bands in the absorption spectrum of tyrosine have been found.
3. The absorption spectra of horse and human serum proteins have been measured : the values obtained have been found to be in very close agreement.
4. The ratio $\frac{\text{extinction coefficient at head of curve}}{\text{extinction coefficient at foot of curve}}$ may be taken as an index of purity of a given sample of protein.

5. It has been shown that error is not introduced by "scattering" of the radiation due to the colloidal condition of the proteins.

6. A method of determining the ratio of albumin to globulin in a mixture of the two is suggested.

The writer is indebted to Prof. H. E. Roaf, Dr. J. Marrack and Dr. W. A. M. Smart for their advice and assistance. Special thanks are due to Dr. L. F. Hewitt for the preparation of the exceptionally pure samples of protein used in this investigation; also to Messrs. Adam Hilger, Ltd., for providing the special spectrometer employed in the diffusion experiments.

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Cytochrome and Respiratory Enzymes.

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I.—INTRODUCTION.

The object of this paper is the study of the functional relationship between the intracellular hæmatin compounds and the oxidising enzymes such as dehydrases and oxidases. It was shown previously (1925–1927) that aerobic organisms contain a very widely distributed intracellular hæmatin pigment—cytochrome—which can undergo reversible oxidation and reduction without

being destroyed. Being the only compound directly visible in the living cell, cytochrome gives us important indications, not only of its own activity but also of that of other components of the respiratory system of the cell.

The present paper will first deal with the thermostable peroxidase of yeast and other cells, and with the true thermolabile oxidases such as the indophenol oxidase of yeast and muscle cells and the polyphenol oxidase of potato. This will be followed by the study of intracellular hæmatin compounds, and especially of the effects of various factors on the oxidation and reduction of cytochrome. The results of this study will enable us to determine the nature of the relationship between the oxidising enzymes and the intracellular hæmatin compounds, and this will help to elucidate at least one portion of the complicated respiratory mechanism of the cell.

II.—THERMOSTABLE PEROXIDASE IN YEAST CELLS.

A.—*Historical.*

Schönbein (1863a) found that yeast does not blue guaiacum in the presence of H_2O_2 . Tolomei (1896), Grüss (1901) and Issaiew (1904) found in yeast a small amount of an enzyme oxidising various polyphenols. More recently, Harden and Zilva (1914) have shown that fresh yeast, or washed, dried and acetone yeast, contain a peroxidase which oxidises paraphenylenediamine in the presence of H_2O_2 . They could not detect, however, the presence of a real oxidase, as neither paraphenylenediamine nor benzidine was oxidised in the absence of H_2O_2 . Bach (1916) repeated their experiments, but came to the conclusion that yeast cells are completely devoid of either true oxidase or peroxidase. He explains the positive results obtained by previous authors as being due to acids or acid salts present in their yeast preparations.

These few references comprise the whole literature on the oxidases of yeast.

B.—*Personal Observations.*

Freshly prepared 5 per cent. suspension of Delft bakers' yeast treated with an alcoholic solution of benzidine or guaiacum in the presence of H_2O_2 does not give a distinct peroxidase reaction. The benzidine and guaiacum remain colourless or turn slightly blue, while the H_2O_2 is rapidly decomposed by the powerful catalase of yeast cells. Similar results are obtained with suspensions of dry or acetone yeast. If, on the other hand, the suspensions of fresh, dried or acetone yeast, are boiled or even autoclaved and cooled, they strongly oxidise benzidine, guaiacum and paraphenylenediamine in the presence of

H_2O_2 . A positive peroxidase reaction is also obtained, as was shown by Harden and Zilva, with a suspension of dry or acetone yeast thoroughly washed in water. This shows that boiling or washing the yeast cells destroys or removes the substances which inhibit the peroxidase reaction of untreated cells. Heating the cells may have two effects: it destroys the reducing systems which inhibit the peroxidase reaction, and it makes the reagent more accessible to the peroxidase. Washing the dried cells removes the soluble compounds of the reducing systems.

As to the substances responsible for the thermostable peroxidase reaction in yeast, we have sufficient evidence to show that they belong to the iron-porphyrin compounds present in the cells. We have seen previously (Keilin, 1925, 1927) that yeast cells contain at least four hæmatin compounds: (1) an unbound protohæmatin, and three hæmatin compounds, a' , b' , c' , of cytochrome. On the other hand, it is well known that hæmoglobin, and all its derivatives which contain iron-porphyrin, oxidise guaiacum and benzidine in the presence of hydrogen peroxide, and that this property is thermostable. The component c' of cytochrome, which can be extracted in an unmodified form, gives a very distinct peroxidase reaction in a dilution which, when examined with the spectroscope, hardly shows an absorption band in a layer 100 mm. thick; while fresh yeast compressed between two slides shows the band c in a layer 0.2 mm. thick. Yeast cells with their four hæmatin compounds are bound therefore to give a positive peroxidase reaction provided there are no substances, or systems in the cells inhibiting this reaction. If, however, the peroxidase property of the hæmatin compounds cannot be demonstrated in living yeast cells it does not mean that it is not functional. Although the oxidation of artificial substances which never occur in cells such as benzidine and guaiacum is inhibited, the hæmatin compounds may still display their peroxidase activity towards substances which occur naturally in cells.

III.—THERMOSTABLE PEROXIDASES IN OTHER ORGANISMS.

Hæmatin compounds are undoubtedly responsible for the peroxidase reaction in bacteria. It is known that while the aerobic bacteria (*Bacillus subtilis*, *B. proteus*, *B. megatherium* and others) which contain a thermostable peroxidase (Callow, 1926) are rich in cytochrome and other hæmatin compounds, the anaërobic bacteria (*B. sporogenes*, *Streptococcus acidilactici*), which do not give a peroxidase reaction, are completely devoid of hæmatin compounds. Even in higher plants the peroxidase reaction is often due to the hæmatin

compounds present in their cells, especially when the peroxidase is thermostable.

In animal tissues (vertebrates and invertebrates) I have found invariably that the intracellular substances responsible for the peroxidase reaction are thermostable, and this applies to all organisms, both with and without hæmoglobin. Moreover, as in the case of bakers' yeast so in animal tissues, a positive peroxidase reaction is often obtained only after boiling the tissue, and the intensity of the reaction corresponds to the hæmatin and cytochrome content of these cells.

Fresh wing muscles of insects, for instance, do not blue guaiacum or benzidine in the presence of H_2O_2 ; but, after boiling, they give a very powerful peroxidase reaction. Even the eggs of *Ascaris megalocephala* after heating give a very strong peroxidase reaction within their nuclei and the perinuclear protoplasm. In other cases, such as the cells of various organs of snails, which are also rich in hæmatin compounds, a positive peroxidase reaction may be obtained even without heating.

We may say therefore, that in all the organisms examined, cytochrome and other hæmatin compounds are responsible for the thermostable peroxidase reactions. As, on the other hand, these compounds are almost universally distributed, the thermostable peroxidase must have a much wider distribution than is revealed by means of a few artificial reagents, such as benzidine and guaiacum.

IV.—INDOPHENOL OXIDASE IN YEAST CELLS.

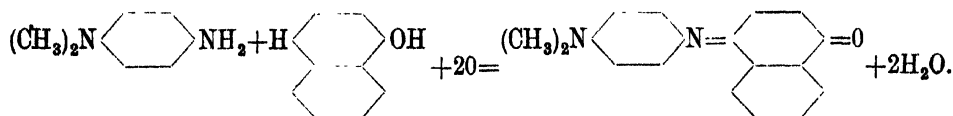
A.—*Effect of Various Factors on the Indophenol Reaction.*

In addition to the hæmatin compounds which show the properties of a thermostable peroxidase, yeast cells also contain a powerful thermolabile oxidase system. The presence of this oxidase, which, as we have seen, was denied by Harden and Zilva (1914) and Bach (1916), can be demonstrated easily with appropriate methods.

The best reagents for revealing this oxidase are: (1) a neutralised 1 per cent. solution of paraphenylenediamine hydrochloride; or (2) the "Nadi" reagent, composed of equal parts of M/100 solutions of dimethylparaphenylenediamine hydrochloride and alpha-naphthol in 50 per cent. alcohol, and of 0.25 per cent. aqueous solution of sodium carbonate. All the reagents have to be freshly prepared before use.

Paraphenylenediamine on oxidation gives a dark purple meri-quinonoid

salts, while "Nadi" reagent, which is one of the modifications of Röhman and Spitzer's reagent, oxidises to indophenol blue :



These reagents, especially when mixed with slightly alkaline phosphate buffer (pH 7.3) and left in air, gradually undergo an autoxidation. It was shown by Wertheimer (1926) that this autoxidation is due to the presence of traces of heavy metals such as iron or copper. In fact, it is accelerated by the addition of various salts of these metals, while it is inhibited with traces of potassium cyanide. The gradual autoxidation of these reagents was often taken as a warning against their validity as oxidase reagents. On the contrary, I have tested these reagents with a great variety of tissues examined under different conditions and I have found them very reliable, especially if they are freshly prepared before use, and if the experiments are carried out with proper controls. Moreover, quantitative experiments with indophenol oxidase carried out in Barcroft's differential manometer, corroborated the results obtained in the test-tube experiments.

The suspensions of yeast cells used for the study of oxidases are prepared in the following way : 25 grammes of fresh Delft bakers' yeast (which corresponds to 7 gms. of dry yeast) are suspended in 50 c.c. of phosphate buffer mixture pH 7.3, giving 72 c.c. of suspension, which we shall call stock A. About 30 c.c. of this suspension are collected in a small Erlenmeyer flask, rapidly warmed to about 52° C. (the temperature of the water-bath must be kept below 57° C.) and kept at that temperature in a thermostat for about 1½ hours. This suspension, which we shall call stock B, when removed from the thermostat, is rapidly cooled to room temperature.

The stock suspensions A and B, which are of the right concentration for spectroscopic examination, are too thick for the oxidase reaction. The most convenient concentration of yeast for indophenol reaction is obtained by re-suspending 10 c.c. of stocks A and B in 100 c.c. of phosphate buffer-mixture, giving the corresponding dilute suspensions of A and B.

The indophenol reaction is carried out in 10 test-tubes of equal diameter, such as buffer-tubes, standing in the same rack. Each of the tubes from 1 to 5 receives 2 c.c. of the dilute stock suspension B, while each of the remaining 5 tubes receives 2 c.c. of the dilute stock suspension A. Two similar tubes with 2 c.c. of dilute suspension A are put in a freezing mixture, reaching a temperature

of -2° to -4° C. The tube B_1 remains untreated ; B_2 is boiled ; B_3 receives M/1000 of KCN ; to B_4 is added a drop of dilute solution of H_2S ; and to B_5 is added $n/30$ of sodium pyrophosphate. Tube A_1 is untreated ; A_2 is boiled ; A_3 receives M/1000 of KCN ; A_4 a drop of H_2S ; and A_5 ethyl urethane (making 5 per cent.). Tubes A_6 and A_7 in the freezing mixture remain untreated. To each of the 12 tubes is then added 0.5 c.c. of freshly prepared "Nadi" reagent and the rack with the 10 tubes (B_1 to B_5 and A_1 to A_5) is shaken for a few minutes. After 6 to 12 minutes' shaking the colour of the suspensions, or in other words, the formation of indophenol blue, in the 12 tubes is carefully compared.

The result of this experiment, which is schematically represented in fig. 1 is as follows. The strongest reaction is obtained in tubes B_1 and B_5 , which

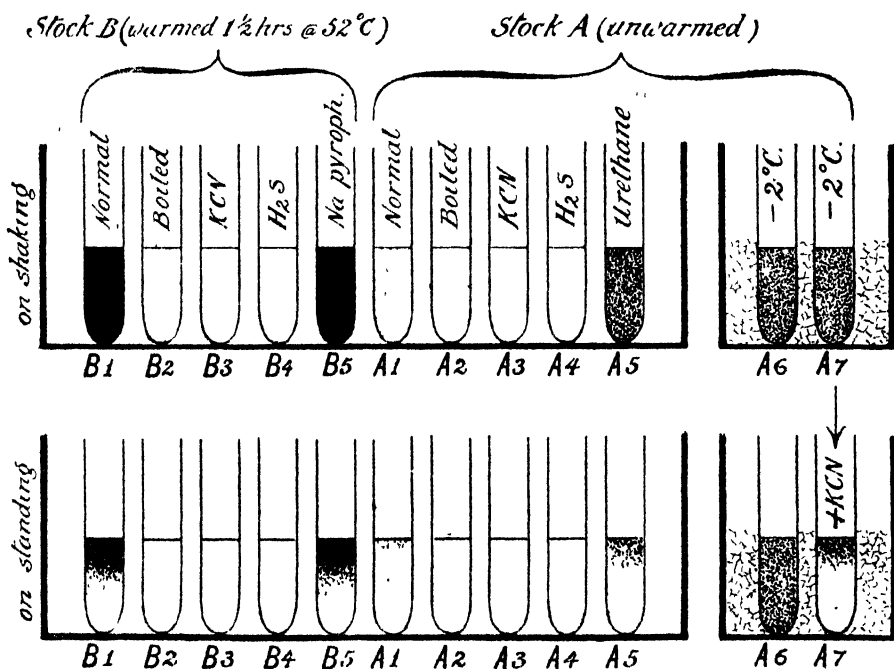


FIG. 1.—Diagram showing the relative strength of indophenol reactions given by yeast suspensions treated in various ways.

show a dark blue colour ; the reaction is strong but less marked in tubes A_5 , A_6 and A_7 ; it is very feeble in tube A_1 ; and negative in B_2 , B_3 , B_4 , A_2 , A_3 and A_4 . The blue colour of the yeast suspension in tubes B_1 , B_5 , A_5 , A_6 and A_7 is due to the granules of indophenol blue deposited within the protoplasm of the yeast cells, which can easily be seen by microscopical examination.

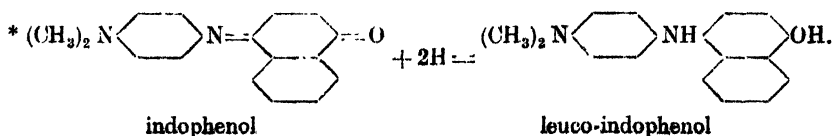
From these experiments, which were repeated many times and always with the same results, we can draw the following conclusions:—

- (1) Yeast cells contain a powerful indophenol oxidase system which is easily revealed by the oxidation of the "Nadi" reagent or of paraphenylenediamine.
- (2) This oxidase is thermolabile, being irreversibly destroyed by boiling or heating to 70° C.
- (3) It is strongly inhibited by KCN and H₂S.
- (4) Sodium pyrophosphate has no effect upon its activity.
- (5) The oxidation of the above reagents with untreated yeast cells is prevented by reducing systems of the cells, so that the positive oxidase reaction is obtained only when the activity of these reducing systems is inhibited by: (a) various narcotics such as urethane; (b) warming the cells for 1½ hours at 52° C.; or (c) lowering the temperature of the cell suspension to -2° C. Positive, but not very strong, indophenol reaction is given also by plasmolysed and washed yeast, and by yeast suspension actively aerated for 1 or 2 hours at a temperature of 37° to 39° C. The indophenol oxidase is on the other hand greatly inhibited by drying the cells or treating them with acetone, as the suspensions of dry yeast or of zymine even washed in water give a feeble indophenol reaction.

The indophenol blue in tubes B₁, B₅, A₁ and A₅, on standing gradually reduces to a leuco-compound or indophenol white* and this reduction proceeds from the bottom of the tube towards the top.

After standing for an hour the yeast cells settle to the bottom of the tube forming a white precipitate, while the supernatant fluid is coloured blue. This fluid can be removed carefully and replaced with fresh water without disturbing the cells. On shaking the tube and resuspending the yeast cells they rapidly regain the dark blue colour they had before the reduction. This shows once more that we are dealing with a reaction taking place inside the protoplasm of the cells.

It shows also that although we have greatly inhibited the reducing power of



the cells in tubes B₁, B₅ and A₅ we have not abolished it. The suspension in tubes A₆ and A₇ does not decolorise on standing, showing that the rate of reduction is slower than that of the diffusion of oxygen and its activation by the oxidase. If, on the other hand, a drop of 1 per cent. KCN is added to A₇ the reduction of indophenol takes place on standing.

The decolorised yeast suspension, on shaking, rapidly oxidises the indophenol white to indophenol blue; but, if a drop of 1 per cent. solution of KCN is added before shaking, the reoxidation of indophenol is not complete. As it is known that the leuco-indophenol is autoxidisable even in the presence of KCN, this experiment shows that the complete oxidation of indophenol white, while the reducing substances are still present, can be accomplished only in the presence of the active oxidase system. The latter either oxidises the leuco-compound directly, or, by oxidising the reducing substances, allows the leuco-compound to undergo complete autoxidation. In fact, the autoxidation of indophenol white formed within the cells easily takes place even in the presence of KCN, provided that the reducing systems are inhibited or destroyed by warming the suspension to 65° C.

The negative, or much delayed, indophenol reaction in untreated yeast is not due to the rapid reduction of indophenol blue to indophenol white, because boiling the yeast, after standing with the reagent, and then shaking it in air, does not bring out the blue colour of indophenol blue. These experiments show also that there is no indication at present that the leuco-compound is an intermediate stage of the indophenol reaction.

B.—*The Effect of Carbon Monoxide on Indophenol Oxidase in Yeast Cells.*

(a) *Experiments in Thunberg Tubes.*—It was found recently by Warburg (1926) that CO in high concentration depresses considerably the oxygen uptake of living yeast cells. While the respiration of yeast cells in 5 per cent. of oxygen and 95 per cent. of nitrogen is normal, when N₂ is replaced by the same amount of CO the oxygen uptake drops by 65 to 71 per cent. The inhibitory effect of CO diminishes with the increase of the partial pressure of O₂. Light greatly diminishes the inhibitory effect of CO on respiration; while in dark 95 per cent. of CO in the presence of 5 per cent. O₂ inhibits the respiration by 71 per cent., in light the inhibition is reduced to about 14 per cent. According to Warburg, O₂ and CO compete in the cell for the same respiratory ferment, which has a much greater affinity for O₂ than for CO. Warburg's results have been extended by Haldane (1927), who studied the effect of CO on the behaviour of a moth (*Galleria*), and upon the germination of cress plants.

As the O_2 uptake of intact cells represents the global result of the activity of several respiratory systems, it was naturally interesting to find which of these systems is directly influenced by carbon monoxide. I have shown recently (1927) that carbon monoxide has a definite inhibitory effect upon the activity of indophenol oxidase in yeast cells and mammalian muscles, as well as upon the polyphenol oxidase of potato and oatmeal flour.

The effect of CO on yeast oxidase can be easily shown with the dilute suspension of stock B (*cf.* p. 210). The reaction is carried out in modified Thunberg methylene-blue tubes (fig. 2). Each tube (T) receives 2 c.c. of yeast

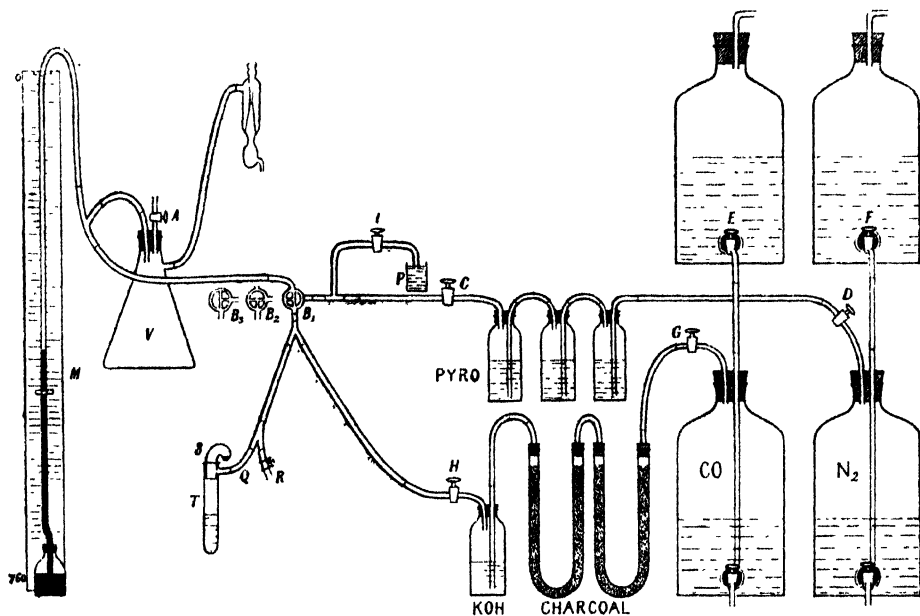


FIG. 2.—Schematic figure of the apparatus used for filling Thunberg tubes and differential Barcroft manometers with various gas mixtures. (For lettering, see text.)

suspension, and 0.5 c.c. of "Nadi" reagent or of paraphenylenediamine is placed in the curved portion of the stoppers. Six of these tubes are fixed in a rack specially made so that it can be reversed holding the tubes in position. The six tubes with yeast suspension and "Nadi" reagent are rapidly filled with the required gas mixtures by means of the apparatus shown in fig. 2. The tube, T, with its stopper open, is connected with the rubber tube Q, all the taps of the apparatus being closed. The three-way tap B is then turned in the position B_3 ; taps E and G are opened; and the tube is exhausted by means of a water pump until the mercury in the graduated pressure gauge M reaches the

desired pressure. The tap B is then turned in the position B₂, and tap H is opened, allowing the carbon monoxide to pass through two U-tubes of absorbing charcoal and a flask of 20 per cent. KOH, and to fill the tube T. The tap H is closed, the stopper S of the Thunberg tube is turned in the opposite direction, and the tube is disconnected. In a similar way the tube is filled with nitrogen. During this manipulation, the taps H, G and E are closed; while F, D and C are opened. The gas for mixing is kept in Mariotte bottles, the reservoir with N₂ is filled directly from a gas cylinder, while the CO is prepared in the usual way from sulphuric and formic acids, and purified by passing through a strong solution of NaOH and through sulphuric acid.

According to requirements, the gases in one of the reservoirs were composed of either pure CO, or of various mixtures of this gas with O₂. Using this method, the whole process of filling six Thunberg tubes with six different gas mixtures can be completed in 6 to 8 minutes. Although the manometric gauge gave sufficient indication as to the composition of the gas mixtures, in several experiments the gas mixtures were analysed in Haldane gas-analysis apparatus.

The first series of reactions was arranged to demonstrate rapidly, by means of a colour reaction, the effect of different concentrations of CO on the intensity of the oxidase reaction. Six Thunberg tubes with yeast suspension and "Nadi" reagent are rapidly filled with various gas mixtures; shaken for 2 minutes in the dark; reversed in order to mix the yeast cells with the reagent; shaken for another 5 to 6 minutes; and then examined to compare the relative intensities of the colours, or, in other words, the relative velocity of indophenol formation.

The results of such experiments are shown in Tables I and II.

Table I.

Tubes.	O ₂ .	CO.	N ₂ .	Reaction.
	Per cent.	Per cent.	Per cent.	
1	7.3	63.4	29.3	XX
2	5.2	73.9	20.8	X
3	3.6	82	14.4	O
4	3.4	0	96.6	XXXX
5	1.8	0	98.2	XXX
6	1.1	0	99	XX

Table II.

Tubes.	O ₂ .	CO.	N ₂	Reaction.
	mm.	mm.	mm.	
1	60	474	240	XX
2	40	574	160	X
3	20	674	80	0
4	30	0	744	XXXX
5	20	0	754	XXX
6	10	0	764	XX

These experiments, which were repeated many times and always gave similar results, show that carbon monoxide combines with the indophenol oxidase of yeast forming an inactive compound incapable of oxidising the phenylene-diamine reagents. They show also that the oxidase has a much greater affinity for O₂ than for CO, as the inhibition is obtained only at very high concentrations of CO, and a negative reaction after 5 minutes' shaking is obtained only when the ratio between CO and O₂ reaches the value of 24.

The inactive compound formed by the combination of oxidase with carbon monoxide can be rapidly reactivated, either by the admission into the tubes of more O₂, or by exposing them to light. Light dissociates the CO-oxidase compound, liberating the active oxidase. This can be easily demonstrated by preparing six vacuum tubes with yeast suspension and "Nadi" reagent and filling them with the same gas-mixture, composed of 6 per cent. O₂, 70 per cent. CO, and 24 per cent. N₂. The tubes are tightly fitted into a rack which is divided into two compartments each holding three tubes. One compartment of the rack is covered with a well-fitting blackened tin box, so that three tubes are kept in dark, while the remaining three tubes are exposed to light. The rack (fig. 3) is shaken for 1 to 2 minutes in front of an electric half-watt fila-

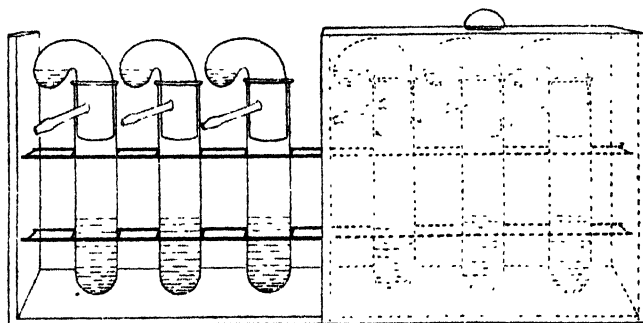


FIG. 3.—Rack with six Thunberg tubes for oxidase reaction under various gas mixtures. Three tubes are exposed to light while the other three are kept in dark under a tin box.

ment lamp of 50 c.p. It is then reversed, the yeast suspension being mixed with the reagent and again shaken in front of the lamp. After 6 to 8 minutes' shaking the three tubes kept in dark are uncovered, and, on comparing the reaction in all six tubes, it is found that while in the three tubes exposed to light the indophenol reaction is strongly positive (XXXX), in the tubes kept in dark the blue colour of indophenol is hardly perceptible (X).

(b) *Experiments in Barcroft Differential Manometers.*—The above experiments clearly demonstrate the effect of CO on indophenol oxidase of yeast, as well as the influence of light on the dissociation of the CO-oxidase compound. They do not give precise information as to the relative affinity of the oxidase for CO and oxygen. Quantitative results can be obtained, however, by estimating the oxygen uptake of yeast oxidase in the presence of the reagents.

These experiments are carried out by means of Barcroft's differential apparatus. The right-hand flask of the apparatus receives 3 c.c. of the dilute yeast suspension of stock B (warmed for $1\frac{1}{2}$ hours at 52° C., and containing 90 mg. of yeast), in phosphate buffer mixture of pH 7.3: 0.3 c.c. of 10 per cent. NaOH is put in a small tube fused to the bottom of the flask; and another short tube, provided with a platinum hook and containing 0.3 c.c. of neutralised solution of paraphenylenediamine hydrochloride (=21 mgs.), is hung from the top of the NaOH tube (fig. 4). The left-hand control flask of the apparatus receives 3 c.c. of phosphate buffer-solution pH 7.3: 0.3 c.c. 10 per cent. NaOH; and the tube with 0.3 c.c. of paraphenylenediamine.

The Barcroft apparatus containing the necessary solutions is then filled with the required gas mixtures in the following manner. The flasks of the apparatus are immersed in a bath, the water of which is kept thoroughly mixed, and the free ends of the manometer tube are connected, by means of rubber pressure tubing Q and R, with the gas-filling apparatus previously described (fig. 2). For these experiments it was found more

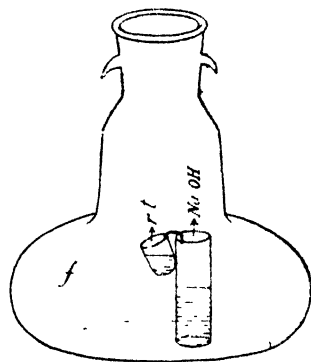


FIG. 4.—Flask (f) of a differential Barcroft manometer showing the NaOH tube and the reagent tube (rt) suspended from the latter.

convenient to prepare beforehand the appropriate gas mixtures in the Mariotte bottles. The Barcroft apparatus is carefully washed out with the required gas mixture by three successive evacuations and fillings. After the final filling of the apparatus, the gas supply is shut off, and by opening

the tap I the pressure within the apparatus is equilibrated with that of the atmosphere. The taps of the Barcroft apparatus are then immediately closed, and after shaking the apparatus in the bath for 3 to 5 minutes and taking the first reading of the manometer, the small reagent tubes are dropped to the bottom of the flasks, the paraphenylenediamine being mixed with yeast suspension (right flask), or with the buffer solution (left flask). The apparatus, with its flasks immersed in a covered bath, is regularly shaken during the whole time of the experiment, and readings are taken every 5, 10 or 15 minutes.

A typical experiment of this series consisted in preparing four differential manometers (I to IV), of which 3 (I to III) received all the necessary solutions, while the fourth (IV), was left without the paraphenylenediamine tubes in order to see the rate of O_2 uptake by yeast suspension warmed to $52^\circ C$. The first apparatus and the fourth were left filled with air; the second was filled with a gas mixture of 1 $O_2 + 4 CO$, and the third with a gas mixture of 1 $O_2 + 8.5 CO$. After mixing the yeast suspension with the reagent the oxygen uptake in all four manometers was noted every few minutes. The results of this experiment, which are represented graphically in fig. 5, clearly demonstrate

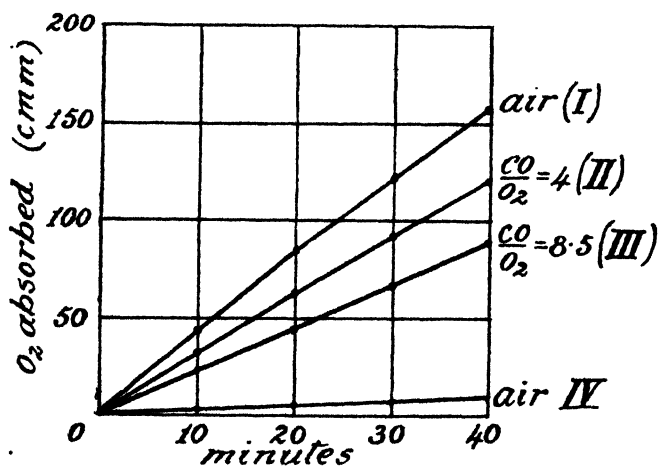


FIG. 5.—Effect of CO on indophenol oxidase of yeast (warmed to $52^\circ C$). Curves I, II and III represent the oxygen uptake of the suspension at $17^\circ C$. in the presence of the reagent (paraphenylenediamine). Curve IV shows the oxygen uptake of the suspension without the reagent.

the inhibitory effect of CO upon the oxidase activity of yeast cells. They also show that the oxygen uptake of yeast suspension of stock B (p. 210) in the presence of paraphenylenediamine is almost entirely due to the catalytic oxida-

tion of this reagent by the oxidase. The oxygen uptake by yeast itself (IV, air, fig. 5) which represents the remains of initial respiration, hardly amounts to 6.5 per cent. of the oxygen uptake of yeast in the presence of the reagent, and the slight oxygen uptake due to the autoxidation of the reagent is completely eliminated, as the same amount of reagent was introduced into the left flask of the manometer.

The partition of oxidase between O_2 and CO, or, in other words, the relative affinity of the oxidase for O_2 and CO, can be calculated easily by applying here the following equation used by Warburg: $\frac{n}{1-n} \cdot \frac{CO}{O_2} = K$, where the residual respiration n is equal to $\frac{A}{A_0}$, A being the oxygen uptake in CO mixture and A_0 the oxygen uptake in air; the respiratory inhibition $1 - n = \frac{A_0 - A}{A_0}$.

Taking as an example the numerical results in one of the experiments, where 3 c.c. yeast suspension (= 90 mg. yeast) + 0.3 c.c. paraphenylenediamine (= 21 mg.) at $15^\circ C$. in 10 minutes took up 150 c.mm. of O_2 in air (= A_0); 108 c.mm. in gas mixture 1 O_2 + 3.8 CO (= A); the partition constant K is easily calculated: $K = \frac{108}{150 - 108} \times 3.8 = 9.8$. The results of other experiments are given in Table III.

These experiments show that the inhibition of oxidase activity with CO is approximately proportional to the partial pressure of this gas. When the concentration of CO is about four times that of oxygen the oxidase activity is inhibited by about 15 to 25 per cent. When the concentration of CO reaches nine times that of O_2 the inhibition increases to 35 to 54 per cent. They also show that both CO and O_2 compete for the same component of oxidase, which has a much greater affinity for O_2 than for CO.

Table III.

Exp. No.	t C.	Yeast, c.c.	Time, mins.	Gas volume percentage.		CO O ₂	O ₂ uptake c.mm.	Per-centage of in-hibition.	$K = \frac{n}{1-n} \cdot \frac{CO}{O_2}$
				O ₂ .	CO.				
1	16	3	20	air		0	71	0	—
				11.5	44.5	3.8	57	20	15.2
				10.5	89.5	8.5	41	42	11.8
2	15	3	40	air		0	150	0	—
				11.5	44.1	3.8	108	28	9.8
				10.5	89.5	8.5	78	48	9.2
3	16	3	10	air		0	40	0	—
				11.5	44.1	3.8	29	27	10.0
				10.5	89.5	8.5	21	48	9.2
4	17	3	22	air		0	70	0	—
				19.6	79.6	4.1	60	14	24.5
				10	90	9	51	27	24.4
5	17	2.5	16	air		0	63	0	—
				19.5	79.5	4	47	25	12
6	17	2.5	26	air		0	105	0	—
				20	80	4	86	18	18.2
7	17	1	25	air		0	40	0	—
				20.7	79	3.8	34	15	21.5
8	19	2	28	air		0	85	0	—
				19.5	79.5	4	68	20	16
9	19	2	33	air		0	71	0	—
				10	90	9	38	47	10.2
10	37.5	2	19	air		0	108	0	—
				20.7	79	3.8	90	17	18.6
				10	90	9	70	35	16.8
11	37	3	20	air		0	230	0	—
				20	80	4	172	25	12
				10	90	9	120	48	9.8

Indophenol oxidase in yeast.—Inhibition of oxidase activity with CO; 1 c.c. of yeast suspension contains approximately 30 mgs. of yeast. In each case the volume of suspension was brought up to 3 c.c. with phosphate buffer pH 7.3. Each flask received 0.3 c.c. of neutralised para-phenylenediamine hydrochloride (21 mgs.).

C.—General Properties of Indophenol Oxidase of Yeast Cells.

If we compare the above results with those obtained by Warburg on the total respiration of yeast cells we find that CO inhibits the activity of the oxidase to about the same degree as it inhibits the normal respiration of intact cells. The only difference which can be noticed consists in the effect of tempera-

ture on this inhibition. While a rise of temperature increases the inhibitory effect of CO on respiration, it has very little or no effect on the CO inhibition of indophenol oxidase (fig. 6). The oxidase activity and the total respiratory

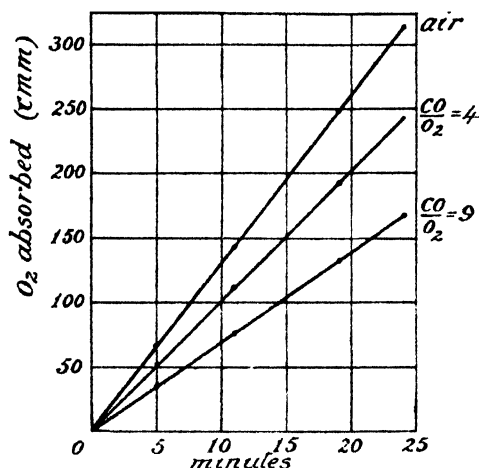


FIG. 6.—Effect of CO on the indophenol oxidase of yeast suspension at 37° C.

activity of yeast cells differ also in their behaviour towards narcotics such as urethane. While 0.57 M. solution of ethyl urethane depresses the total oxygen uptake of untreated yeast cells by at least 68 per cent. it has no effect on the oxygen uptake of yeast cells (warmed to 52° C.) in the presence of paraphenylenediamine. The significance of these two properties of oxidase will be discussed later after dealing with the oxidase systems of other cells.

It must be kept in mind that indophenol oxidase is not soluble and does not diffuse outside the cells, so that the oxidation of the reagents takes place only when they penetrate into the protoplasm of the cells. Moreover, when the reagent is oxidised it usually remains precipitated within the protoplasm, and ultimately inhibits the activity of the oxidase, presumably by preventing the access of fresh reagent to the oxidase system.

Summarising the above results we can say that the activity of indophenol oxidase of yeast cells is inhibited by KCN, H₂S and CO in the same way as the respiratory activity of these cells, it is greatly reduced by drying the cells or treating them with acetone or alcohol, and completely abolished by heating the cells to 70° C.

Numerous attempts to extract the oxidase enzyme from plasmolysed, frozen or dried yeast cells failed completely. It was even difficult to obtain a preparation of yeast cells with the oxidase exposed on the surfaces, as only a small

proportion of cells treated in various ways were found with their walls damaged or broken. Preparations with oxidase surfaces exposed, which will be described presently, can be obtained only from other types of cells, such as those of heart muscle.

V. INDOPHENOL OXIDASE IN HEART MUSCLES.

Method of Preparation.—About 30 to 40 gms. of fresh sheep's heart, cleared of fat and ligaments, and finely minced, are shaken up in 1 litre of tap water. After 10 minutes' standing the suspension is pressed out through linen, re-suspended in 1 litre of water, thoroughly shaken for 5 minutes, and left standing for another 10 minutes. The suspension is then pressed through linen, mixed with an equal volume of washed sand, thoroughly pounded in a mortar and suspended in 200 c.c. of phosphate buffer mixture pH 7.3. The sand rapidly settles and the thick muscle suspension is poured off into another vessel. The muscle suspension prepared in this way shows a feeble α -band of oxyhæmoglobin, and the particles are sufficiently small to pass through a fine pipette.

This stock suspension is too thick for the study of oxidase, and is usually diluted to contain not more than 20 to 25 mgs. of wet weight of muscle per 1 c.c. of suspension. The *main properties* of this preparation are as follows :—

(1) The oxidase activity of the same muscle preparation remains fairly constant for several days, provided the suspension is kept at a low temperature, on ice for instance. The strength of the oxidase varies, however, with the preparation as it is difficult to obtain two suspensions containing tissue particles of the same size. In the results given below only experiments carried out simultaneously on the same muscle preparation were considered and compared with each other, in order to determine the degree of inhibition or the value of K.

(2) Without any further treatment, in ordinary test-tube experiments, the dilute suspension of this preparation rapidly gives a very strong indophenol reaction.

(3) The oxidised dye developed on shaking does not reduce to the leuco-compound on standing, which shows that the muscle suspension is devoid of the reducing substances which are present in unwashed muscle.

(4) The oxygen uptake of the suspension itself, measured in differential manometers, is insignificant and represents only 1 per cent. of the oxygen uptake of the same suspension mixed with paraphenylenediamine.

(5) M/1000 KCN or H_2S inhibits completely the indophenol reaction, as well as the oxygen uptake of preparations in the presence of paraphenylenediamine.

(6) Ethyl urethane 5 per cent. ($= 0.57$ M) and sodium pyrophosphate N/30 have no effect on the activity of the oxidase. The oxygen uptake is inhibited only by 18 per cent. with N/15 sodium pyrophosphate, and by 11 per cent. with N/6 NaF (Table IV).

Table IV.

No.	Muscle, c.c.	Time, mins.	Narcotic.	O ₂ uptake, c.mm.
1	2.5	70	0	125
1	2.5	70	5 per cent. urethane	147
2	2.5	46	0	82
2	2.5	46	5 per cent. urethane	77
3	2.5	30	0	178
3	2.5	30	N/30 Na. pyrophosphate	177
3	2.5	30	N/6 NaF	158
4	1.5	53	0	188
4	1.5	53	N/15 Na. pyrophosphate	153

(7) Carbon monoxide in dark distinctly inhibits the activity of indophenol oxidase of muscle. This can be easily demonstrated with the previously described Thunberg tubes experiments. As in yeast, the oxidase of muscle inhibited with CO in dark, is reactivated when exposed to light. The experiments on the effect of CO on the oxygen uptake by the muscle preparation containing paraphenylenediamine, were carried out in differential manometers in the same manner as was previously described for yeast. The results of these experiments, which are summarised in fig. 7 and Table V, show that carbon

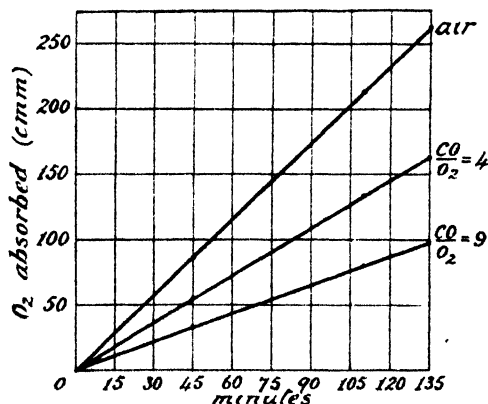


FIG. 7.—Effect of CO on the indophenol oxidase of heart muscle.

monoxide seems to have a greater inhibitory effect upon the indophenol oxidase of muscle than upon that of yeast. In yeast preparations, when the

Table V.

Exp. No.	t C.	Muscle, c.c.	Time, mins.	Gas volume percentage.		$\frac{\text{CO}}{\text{O}_2}$	O_2 uptake c.mm.	Per-centage of inhibition.	$K = \frac{n}{1-n} \cdot \frac{\text{CO}}{\text{O}_2}$
				O_2	CO.				
1	17	3	110	air		0	226	0	---
				11.5	44.1	3.8	135	40	5.7
				10.5	89.5	8.5	81	64	4.8
2	16	3	60	air		0	113	0	---
				11.5	44.1	3.8	51	55	3.1
				10.5	89.5	8.5	40	65	4.6
3	18	2.5	55	air		0	272	0	---
				20	80	4	169	38	6.5
				10	90	9	112	59	6.3
4	18	2.5	23	air		0	114	0	---
				20	80	4	71	38	6.6
5	18	2.5	28	air		0	152	0	---
				10	90	9	61	60	6.0
6	19	1.5	35	air		0	116	0	---
				20	80	4	77	34	7.8
				10	90	9	46	61	5.8
7	19	1.5	65	air		0	200	0	---
				20	80	4	123	38	6.5
				10	90,	9	81	59	6.2
8	38.5	1.5	40	air		0	278	0	---
				20	80	4	215	23	13.4
9	38.5	1.5	30	air		0	224	0	---
				10	90	9	137	39	14.0
10	38.5	1	30	air		0	147	0	---
				10	90	9	99	33	18.2

Indophenol oxidase of heart muscle.—Inhibition of oxidase activity with CO. 1 c.c. of muscle suspension contains approximately 25 mgs. wet weight of muscle. In each case the volume of suspension was brought up to 3 c.c. with phosphate buffer pH 7.3. Each flask received 0.3 c.c. of neutralised paraphenylenediamine (\approx 21 mgs.).

concentration of CO was about nine times that of O_2 , the oxygen uptake was reduced by 30 to 54 per cent.; while in muscle suspension, in the same concentration, the oxygen uptake is reduced by 58 to 64 per cent., and the value of K (constant of partition of oxidase between O_2 and CO) is reduced to 3.2–6.5 (average value = 5.6).

It is possible, however, that in muscle preparation the oxidase is more accessible to the reagent than in yeast, where it has to diffuse through cell walls. The rate of this diffusion may become sufficiently slow to prevent the complete

saturation of the enzyme with the substrate. We know, on the other hand, that at every partial pressure of CO the optimum inhibition can only be obtained when the enzyme is fully saturated with substrate.

(8) Experiments 8, 9 and 10 (Table V) show that with the rise of temperature from 19° to 38.5° C. the inhibition produced by CO diminishes and the average value of K increases from 5.6 to 15.5.

Attempts to extract the indophenol oxidase from the muscle preparation also failed. An opalescent fluid can be obtained which gives a fairly good indophenol reaction, but on careful examination it was found that the reaction was given actually by very small microscopic particles. On successive filtrations the fluid becomes clearer and at the same time gradually loses the property of oxidising the paraphenylenediamine.

VI.—POLYPHENOL OR CATECHOL OXIDASE IN POTATO.

Having failed to extract the indophenol oxidase from yeast and from muscles, it was important to find whether an oxidase from other tissues could be obtained in a clear solution, in order to compare its properties with those of the indophenol oxidase previously described. Such a preparation can be obtained, and the best example of it is the polyphenol oxidase of potato. A great number of higher plants, especially those which show discolouration on injury, blue guaiacum directly. They possess a direct oxidase system, which according to M. W. Onslow (1920) is composed of three components: two alcohol insoluble enzymes, oxygenase and peroxidase, and an alcohol soluble "catechol substance" or an aromatic compound with two hydroxyl groups in the "ortho" position. The inter-reaction between oxygenase and catechol leads, according to Onslow, to the formation of a peroxide, which being decomposed by peroxidase liberates active oxygen, which can oxidise guaiacum and other substances.

It was shown more recently, however, by Szent-Györgyi (1925) that the main product of the action of oxygenase upon catechol is not a peroxide, but an orthoquinone, which will blue guaiacum directly, in the absence of both oxygenase and peroxidase. These results have been confirmed by Onslow and Robinson (1926) and by Pugh and Raper (1927). The enzyme which was described by Bach and by Onslow as oxygenase has, as we shall see presently, all the essential properties of a typical oxidase, and we shall refer to it in this paper as polyphenol or catechol oxidase. A very good sample of this enzyme was kindly prepared for me by Dr. Szent-Györgyi, who also supplied me with the following description of the method he used for its preparation:—

"The potatoes are washed and dried. The peripheral portion is peeled off to a depth of $\frac{1}{2}$ to 1 cm.; the central part being discarded. The peels are rapidly minced, falling from the mincer directly into three times their volume of 96 per cent. alcohol. The minced material is uniformly suspended in alcohol, and, after standing for half-an-hour, is filtered on a Buchner filter. The residue is washed with fresh alcohol, which is then thoroughly sucked off. To every 100 gms. of residue 100 c.c. of water are added and the whole well mixed. After half-an-hour the suspension is filtered through muslin, the residue being pressed out in a press. The fluid is filtered through paper pulp and mixed with three times its own volume of alcohol. The precipitate is separated by centrifuging and resuspended in $\frac{1}{4}$ of the previous volume of water. The suspension is centrifuged, the clear supernatant fluid is poured off, mixed with three times its volume of alcohol, centrifuged again, the precipitate washed with alcohol-ether and ether, collected and dried in vacuum."

Several samples of oxidase prepared by this method gave very good results, although the strength of the oxidase varied with the sample. Such oxidase preparations can be kept for several months, but their activity gradually decreases. The *main properties* of the polyphenol oxidase of potato prepared by Szent-Györgyi's method are as follows:—

(1) In the dry state it is in the form of a light grey powder which is very soluble in water or buffer mixtures, giving a clear, pale yellowish solution.

(2) It very rapidly oxidises catechol, which on oxidation gradually turns yellow, pink, brown and, finally, dark brown.

(3) It does not give a peroxidase reaction with benzidine, guaiacum or other reagents in the presence of hydrogen peroxide.

(4) It oxidises paraphenylenediamine or "Nadi" reagent only in the presence of catechol. In other words, it does not give a direct indophenol reaction.

(5) The oxidation of catechol and of other reagents by this enzyme is strongly inhibited by M/1000 KCN and by H_2S , and is completely abolished by heating the solution to 70°C .

(6) The oxidation of catechol is greatly inhibited by carbon monoxide, which can be easily seen from the rate of development of colour in Thunberg tubes experiments carried out in the same way as was previously described.

(7) Light has no effect on CO inhibition of catechol oxidase. The compound formed between catechol oxidase and carbon monoxide does not seem to be dissociated by light. The rate of catechol oxidation by the oxidase was the same in glass Thunberg tubes or in quartz tubes filled with the same gas mixtures

and kept in dark or exposed to light, even as strong as that of an exposed arc.

(8) Quantitative experiments on the effect of carbon monoxide on the oxygen uptake by polyphenol oxidase in the presence of catechol were carried out in the usual manner in the Barcroft differential manometers. The right flask received 2.7 c.c. of oxidase solution in phosphate buffer-mixture (pH 7.3) containing from 3 to 24 mgs. of oxidase preparation; a small tube with 0.3 c.c. of catechol solution (= 7 mgs. of re-sublimated catechol), and 0.3 c.c. of 10 per cent. KOH in the tube fused to the bottom of the flask. The left (control) flask received 2.7 c.c. of buffer-mixture; a small tube with 0.3 c.c. of catechol, and 0.3 c.c. of KOH.

The results of a number of these experiments, which are given in fig. 8 and Table VI, show that carbon monoxide has a much greater inhibitory effect

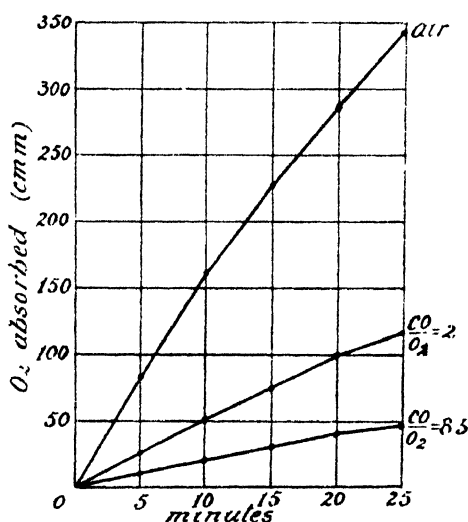


FIG. 8.—Effect of CO on the polyphenol or catechol oxidase of potato.

upon the activity of the polyphenol oxidase of potato than upon the indophenol oxidase of yeast or muscle. At temperatures varying from 14° to 23° C. carbon monoxide, in concentration four times that of oxygen, inhibits the oxygen uptake by 71 per cent., and in concentration nine times that of oxygen, inhibits the reaction by 83 per cent. The average value of K at these temperatures is 1.5. With increase of temperature the degree of inhibition decreases; at 37° to 38° C. the inhibition of oxygen uptake in gas mixtures 1 O₂ to 4 CO, and 1 O₂ to 9 CO, drops to 52 per cent. and 69 per cent. respectively, while K reaches the mean value of 3.95.

Table VI.

Exp. No.	t C.	Oxidase, mgs.	Time, mins.	Gas volume percentage.		$\frac{\text{CO}}{\text{O}_2}$	O_2 uptake c.mm.	Per-centage of in-hibition.	$K = \frac{n}{1-n} \cdot \frac{\text{CO}}{\text{O}_2}$
				O_2 .	CO.				
1	14	24	35	air		0	262	0	—
				11.5	44.1	3.8	68	74	1.3
				21	79	3.8	69	74	1.3
2	14.5	24	25	air		0	304	0	—
				20	42	2.1	108	65	1.2
				10.5	89.5	8.5	36	89	1.1
3	15	24	34	air		0	310	0	—
				11.5	44.1	3.8	96	69	1.7
				10.5	89.5	8.5	58	81	1.9
4	16	24	20	air		0	365	0	—
				11.5	44.1	3.8	136	63	2.3
				10.5	89.5	8.5	90	75	2.8
5	16	24	40	air		0	760	0	—
				11.5	44.1	3.8	173	77	1.1
6	16	24	30	air		0	347	0	—
				19.5	79.5	4	73	79	1.1
7	16	16	20	air		0	308	0	—
				10.5	89.5	8	53	83	1.7
8	16	4.8	13	air		0	76	0	—
				19.5	79.5	3.8	17	78	1.1
9	23	12	21	air		0	230	0	—
				20	80	4	64	72	1.6
				10	90	9	38	83	1.8
10	22	3	22	air		0	59	0	—
				20	80	4	19	68	1.9
				10	90	9	8	86	1.5
11	37	6	20	air		0	88	0	—
				20	80	4	43	51	3.9
				10	90	9	27	69	4.0
12	38	12	12	air		0	153	0	—
				20	80	4	73	52	3.7
				10	90	9	47	69	4.1

Inhibition of catechol oxidase by carbon monoxide, for details of experiment see § 8, p. 227.

VII.—GENERAL CHARACTERS OF OXIDASES.

Comparing now the results obtained with the paraphenylenediamine oxidase of yeast and muscle cells with those obtained by Warburg, Meyerhof and others on the total respiration of the cells, it will be found that the factors such as

KCN, H_2S , CO in dark and CO in light, drying the cells, passing them through acetone or alcohol, inhibit the indophenol oxidase activity to about the same degree as they inhibit the normal respiration of the cells. This clearly shows that the indophenol or paraphenylenediamine oxidase takes an essential part in the normal respiratory activity of the cells, and that this oxidase is identical with the "respiratory ferment" of Warburg. The term indophenol oxidase must be retained, however, in preference to the term "respiratory ferment," which is too wide and which implies that it is the only ferment taking part in the respiratory mechanism of the cell. This, however, is not the case, oxidase in the living actively respiring cell represents only one link in the chain forming the complicated respiratory mechanism, in which several other respiratory ferments and systems are involved and intimately interconnected. Moreover, the oxidase systems such as can be revealed by the oxidation of an artificial chromogen have been known for many years.

Although some of the data found in the literature concerning these oxidases are controversial and even valueless owing to the crude or faulty methods often used in these investigations, there still remains very rich and important material, which will be very helpful in studying the function and nature of these enzymes. The wide distribution of oxidases in aërobic organisms, bacteria, plants and animals, was already shown by Schultze and others. The polyphenol or catechol oxidase, which is more resistant to drying and treatment with alcohol, shares with the indophenol oxidase the properties of being inhibited with KCN, H_2S and CO, and destroyed on warming to 70°C . The activity of this oxidase has not yet been investigated in relation to the respiratory activity of cells containing it.

So far, the activity of an indophenol oxidase under various conditions seems to be parallel with the respiratory activity of the cells. Several important questions remain, however, to be discussed in order to understand the actual rôle played by oxidases in the cellular oxidation and to reconstruct at least one of the possible respiratory mechanisms of normal living cells.

(1) It is, for instance, important to determine whether in a normal untreated cell the oxidase behaves in a manner similar to that revealed by the oxidation of an artificial chromogen.

(2) Is it possible to observe the activity of an oxidase independently from that of other respiratory ferments or systems of the cells?

(3) Why does 5 per cent. urethane, which has no effect upon the oxygen uptake by yeast cells (warmed to 52°C .) or washed muscle in the presence of paraphenylenediamine, inhibit the normal respiration of yeast by 68 per cent.

oxidised hæmatin, unites with it forming methæmoglobin, and, when added to reduced hæmatin, it gives reduced hæmoglobin. Protohæmatin which is a Fe-porphyrin compound, does not combine with other native proteins such as albumin, globulin, edestin, etc. In other words, only one native protein—globin—gives compounds with hæmoglobin properties.

Neutral hæmatin combines with denatured globin and other nitrogen compounds such as histidine, pyridine or nicotine, forming a series of compounds known as parahæmatins (Keilin, 1926), examples of which are kathæmoglobin, oxidised cytochrome and oxyhelicorubin.

Reduced hæmatin readily combines with a great variety of nitrogen compounds such as denatured proteins, glycocoll, nicotine, pyridine, piperidine, hydrazine hydrate, potassium cyanide, ammonium, etc., giving rise to the corresponding hæmochromogens, which, of all the iron-porphyrin compounds, have the strongest absorption bands. This important property was definitely proved and generalised by Anson and Mirsky (1925), who also discovered the existence of a true equilibrium between the reduced hæmatin, the nitrogen compound and the hæmochromogen. To form a hæmochromogen one molecule of reduced hæmatin unites with two molecules of nitrogen compound, such as pyridine (Zeynek, 1920 ; Hill, 1926).

Reduced hæmatin, hæmochromogen and hæmoglobin unite with carbon monoxide forming the corresponding carboxy compounds. The carbon monoxide compounds of reduced hæmatin and hæmochromogen (pyridine) contain one molecule of CO (Hoppe Seyler, 1889 ; Roche, 1926 ; Hill, 1926) ; while in CO-hæmochromogen one of the two nitrogen molecules (pyridine) is replaced by a molecule of CO (Hill, 1926). On the addition of excess of nitrogen compound to a carboxy-hæmochromogen, the CO molecule is displaced by the nitrogen compound thus forming hæmochromogen. CO-compounds of hæmoglobin and its derivatives are dissociated by light (Haldane and Smith, 1896 ; Hartridge and Roughton, 1923 ; Krebs, 1928).

Hæmochromogens readily oxidise in air, and, in strongly alkaline solution become dissociated into oxidised hæmatin and the nitrogen compound ; in neutral solution they do not dissociate and, on oxidation, form parahæmatin compounds.

Hæmatin unites with KCN forming the characteristic cyanhæmatin compound, which, on reduction, gives KCN hæmochromogen. The latter readily oxidises in air to KCN hæmatin. Hæmatin, parahæmatin and methæmoglobin are *ferric* compounds (Küster, 1910 ; Conant, 1923–1926), while reduced hæmatin, hæmochromogen, hæmoglobin and oxyhæmoglobin are *ferrous*

compounds. The reduction of methæmoglobin to hæmoglobin (Conant, 1923-1926; Hill and Holden, 1927) and of hæmatin to reduced hæmatin or pyridine hæmochromogen (Haurovitz, 1927; Hill and Holden, 1927) require one equivalent of hydrogen per atom of iron.

IX.—INTRACELLULAR HÆMATIN COMPOUNDS.

A.—*Cytochrome*.

Cytochrome, as was previously shown, is an intracellular pigment very widely distributed in nature and common to aërobic bacteria, yeast, higher plants and animals. The spectroscopic examination of living cells shows that cytochrome may exist in a reduced and an oxidised state. In the reduced state it shows a characteristic absorption spectrum composed of four bands, *a*, *b*, *c* and *d*, occupying approximately the same position in most of the organisms examined. In the muscle of guinea pigs, for instance, *a*, 6045 Å.; *b*, 5662 Å.; *c*, 5500 Å.; and *d*, 5205 Å. On oxidation the absorption spectrum undergoes a marked change. The four strong bands disappear, being replaced by hardly perceptible, diffuse bands lying approximately at α , 5665 Å., and β , 5287 Å. The highest concentration of this pigment is found in cells capable of active metabolism such as the thoracic wing muscles of flying insects, heart muscles of mammals or birds, pectoral muscles of flying birds, bakers' yeast, and some bacteria.

It was shown previously that cytochrome is not one chemically defined substance, but is a mixture of three independent hæmochromogen-like compounds *a'*, *b'* and *c'*, capable of being oxidised and reduced independently from each other. The bands *a*, *b* and *c* of cytochrome represent the α -bands of the three corresponding compounds, while the band *d* represents the fused β -bands of at least two components (*b'* and *c'*). None of the components of cytochrome combines with CO in living untreated cells. The component *c'* of cytochrome can be extracted with water from dry or acetone yeast or from muscles. In water extract the component *c'* does not combine with CO at the physiological range of pH. It becomes oxidisable at pH 13, but does not combine with CO until the solution is made much more alkaline. On neutralising the solution component *c'* loses its properties of autoxidation and of combining with CO.

B.—*Free Intracellular Hæmatin*.

In addition to cytochrome, or even in its absence, all cells of aërobic organisms contain a free unbound hæmatin similar to the protohæmatin of hæmoglobin (Keilin, 1926, 1927b). This can be easily observed in cells containing little

or no cytochrome, such as the endosperm of cereals, especially in the form of oatmeal flour or wheat flour. The microspectroscopic examination of a thick suspension of flour, even on the addition of a reducer, does not reveal yet the presence of hæmatin, because the absorption spectrum of the latter is very indistinct. If, on the other hand, CO or ordinary coal gas is bubbled through the reduced suspension the appearance of two bands of carboxy-hæmatin can be easily observed. The presence of the free hæmatin can also be revealed by transforming it into a hæmochromogen which is easily formed when pyridine with a reducer ($\text{Na}_2\text{S}_2\text{O}_4$) is added to the suspension of flour. The strong absorption bands of pyridine hæmochromogen thus obtained occupy the same position as those of a typical pyridine hæmochromogen obtained from proto-hæmatin. This indicates that the free hæmatin which can be revealed in cells of all organisms is very similar to, if not identical with, the hæmatin of hæmoglobin. Using a slightly more complicated method (Keilin, 1927b, p. 18), the presence of the free hæmatin may be clearly demonstrated in cells containing cytochrome such as bakers' yeast.

C.—*Hæmochromogen Precursor of Cytochrome.*

It was suggested previously (1926) that cytochrome originates from the free intracellular protohæmatin which is present in all aërobic cells. The first step in the development of cytochrome consists in the formation of a hæmochromogen from the free hæmatin and an undetermined nitrogen compound. This hæmochromogen precursor of cytochrome is very widely distributed in nature, and somewhat resembles the component *b'* of cytochrome as well as helicorubin of snails. In some cells, such as *Bacillus coli* and other facultative aërobic bacteria, it represents the only visible hæmatin compound, which oxidises and reduces within the cells, but does not combine with CO. Apart from bacteria it can be seen easily in the yellow patches of variegated leaves of *Euonymus*, especially in spring and early summer, and also in very young leaves of other plants after the extraction of the chlorophyll.

This hæmochromogen compound with its two characteristic absorption bands is abundant in the cells of various organs of snails and slugs. In some organs, such as the alimentary canal, as one approaches the active muscles of the radula, the spectrum of hæmochromogen merges into that of cytochrome. During larval and pupal development of the ordinary blow-fly the free hæmatin and hæmochromogen are gradually replaced by cytochrome.

Under the influence of repeated oxidations and reductions within the cells this hæmochromogen is gradually modified and gives rise to the three com-

ponents of cytochrome. This supposition is supported by the fact that a solution of hæmochromogen prepared from hæmin crystals and one nitrogen compound such as globin, pyridine or nicotine, after repeated oxidation with potassium ferricyanide and reduction with $\text{Na}_2\text{S}_2\text{O}_4$, displays a four-banded absorption spectrum similar to that of cytochrome. In this experiment potassium ferricyanide can be replaced by a weak solution of H_2O_2 .

The gradual modification of a hæmochromogen spectrum into that of cytochrome is also obtained by ordinary autoxidation of pyridine- or hydrazine hydrate-hæmochromogens exposed to air. The modification begins with the gradual shift of the α -band towards the blue end of the spectrum and the appearance of the band a . That these modifications of the porphyrin portion of the molecules are due to oxidation is shown by the fact that these hæmochromogens when kept in Thunberg tubes in pure nitrogen remain for a long time unmodified.

It may be mentioned here that pyridine hæmochromogen extracted from yeast, which is completely destroyed within a few hours when exposed to air, remains unmodified for weeks if kept in pure nitrogen.

D.—Oxidation and Reduction of Cytochrome in Yeast Cells.

The oxidation and reduction of cytochrome can be very easily observed in living untreated cells of various organisms. It can be seen in the wing muscles of a living moth (*Galleria*), for instance, examined by transparency; in a suspension of finely divided muscles, in a suspension of bacteria (*Bacillus subtilis*), or, still better, in a suspension of ordinary bakers' yeast. A suspension of 25 per cent. of yeast cells in water, on standing, shows clearly the four characteristic bands of reduced cytochrome; on shaking, cytochrome rapidly becomes oxidised and the bands disappear. They soon reappear, however, on standing, as cytochrome rapidly becomes reduced. The state of the pigment as seen with the microspectroscope in living actively respiring cells denotes only the difference in the rates of its oxidation and reduction which are constantly taking place.

Effect of Temperature (-2°C. to 39°C.).—At temperatures between 30° and 39°C. cytochrome, in a 25 per cent. yeast suspension, is so rapidly reduced that even in a suspension vigorously aerated it is difficult to perceive the oxidised state of the pigment. At 15° to 20°C. the oxidation of cytochrome is rapid, while the reduction takes place in 15 seconds.* On lowering the temperature

* To compare the rates of reduction of cytochrome, equal concentrations of yeast suspensions are prepared from a sample of yeast and examined in the same layers.

of the same suspension, the rate of reduction becomes slower, and at -2°C . the reduction takes place in between 70 and 120 seconds. At that temperature yeast suspension kept in a thin layer shows cytochrome in a permanently oxidised state. These reversible effects produced by different temperatures show that lowering the temperature has a much greater inhibitory effect on the reduction of cytochrome than on its oxidation.

The effect of higher temperatures on the oxidation, reduction and structure of cytochrome is shown by the following experiments:—

25 gms. of yeast were added to 50 c.c. of phosphate buffer pH 7.3, forming 72 c.c. of stock A. 40 c.c. of this stock were put in a thermostat regulated to 52°C ., and after $1\frac{1}{2}$ hours the suspension reached a temperature of 48°C . Then 20 c.c. of the suspension were rapidly removed and cooled, forming stock B. The remaining 20 c.c. were left in the thermostat until they reached a temperature of 52°C ., when they were removed and rapidly cooled to form stock C. 2 c.c. of each of these three stocks were put in tubes under the micro-spectroscope and after vigorous aeration of the suspension the reduction of cytochrome was carefully timed. The reduction of cytochrome in stock A (unwarmed) took place in 4, 7, 6, 6, 5, 5 seconds, average 5.5 seconds. In stock B (warmed to 48°C .) reduction took place in 10, 12, 10, 9, 10 seconds, average 10.2 seconds. In stock C (warmed to 52°C .) reduction took place in 45, 46, 48, 49, 48 seconds, average 46.6 seconds.

It is important to note that the time of reduction of cytochrome in these experiments was approximately inversely proportional to the oxygen uptake of these three samples of yeast suspension (Table VII).

Table VII.

Stock.	Yeast, mgs.	Time, mins.	a. O_2 uptake, c.mm.	b. Reduction of cytochrome secs. (average).	$\frac{a \times b}{100}$
A	34	30	112	5.5	6.2
B	34	30	65	10.2	6.6
C	34	30	12.8	46.6	6

The ordinary way of preparing the warmed yeast suspension which was used for the oxidase reactions was slightly different. It consisted in rapidly warming the suspension to 50° – 52°C . in a water-bath at 56°C ., and then placing it in a thermostat at 52°C . for 1 to 2 hours. In a 25 per cent. suspension treated in this way and cooled, the reduction of cytochrome takes place in from 60 to 155 seconds.

Temperatures above 65°C . have a distinctly destructive effect upon cytochrome, components a' and b' gradually disappearing, while c' remains unchanged even on boiling for several minutes. After boiling, c' does not oxidise

on shaking, and behaves exactly like component c' in water extracts of dry or acetone yeast.

Effect of Drying.—100 gms. of fresh bakers' yeast on drying regularly yielded 28 gms. of the dry preparation, which is of brownish colour. A 10 per cent. suspension of this yeast in water or phosphate buffer pH 7·3, on standing shows a distinct spectrum of reduced cytochrome. On shaking the suspension with air, cytochrome oxidises very slowly and incompletely, band a becomes slightly paler, band b disappears, and band c remains almost as strong as before. The reduction of the oxidised portion of cytochrome is also very slow, the component b being reduced only after 15 to 20 minutes' standing. Yeast dried in vacuum or in nitrogen is slightly lighter in colour and shows a more complete oxidation and reduction of cytochrome. All the samples of dry yeast show for many months a more or less normal reduced cytochrome, but in old samples only the component b' oxidises in air.

Effect of Acetone.—Cytochrome, in acetone yeast or zymin prepared in the ordinary way, is very incomplete, and is almost reduced to component c' , which does not oxidise on shaking the suspension with air.

Effect of Washing.—Washing fresh yeast in tap water, distilled water, buffer, or ringer solution, has very little or no effect upon the oxidation and reduction of cytochrome. Washing fresh plasmolysed yeast in 5 per cent. NaCl has no effect upon the oxidation of cytochrome, but it retards the reduction of the oxidised pigment. The oxygen uptake of this yeast represents only 14 per cent. of that of fresh yeast. Dry yeast rapidly washed in water shows normal cytochrome, which is almost completely oxidised on shaking in air, and does not undergo reduction on standing. The oxygen uptake of this yeast preparation is nil. The same applies to washed acetone yeast or zymin.

Effect of KCN, Ethyl Cyanide, Acetonitrile and H₂S.—KCN in a concentration of 10^{-4} M inhibits completely the oxidation of cytochrome. Yeast suspension containing this concentration of KCN, even if actively aerated, shows the four characteristic bands (a , b , c , d) of unmodified reduced cytochrome. Even smaller concentrations of KCN, such as M/100000 or M/200000, delay the oxidation of reduced cytochrome. Ethyl cyanide also inhibits the oxidation of cytochrome, but only when used in much higher concentrations; while acetonitrile even in a concentration of 0·2 per cent. has no effect on cytochrome. H₂S in very low concentration completely inhibits the oxidation of reduced cytochrome. In the case of dry yeast washed in water, KCN inhibits only the oxidation of components a' and c' of cytochrome, and has no effect on the oxidation of component b' .

Effect of Sodium Pyrophosphate.—Sodium pyrophosphate has no effect upon the oxidation of cytochrome; but when used in concentrations higher than N/30 it slightly delays the rate of its reduction.

Effect of Ethyl Urethane.—Ethyl urethane in a concentration of 0.57 M. has no effect upon the rate of oxidation of cytochrome (α' and c'), but retards appreciably its reduction. In a sample of yeast suspension thoroughly aerated, in which the reduction of cytochrome takes place on an average in 17 seconds, after the addition of 0.57 M. of ethyl urethane the reduction is delayed to about 45 seconds. It is interesting to note that the oxygen uptake of the suspension with and without urethane is approximately inversely proportional to the time of reduction of cytochrome.

Table VIII.

Yeast, mg.	Time, mins.	Urethane.	O ₂ uptake, c.mm.	Reduction of cytochrome, secs.	Indophenol reaction.
42	60	0	292	17	X
42	60	0.57 M	93	45	XXXX

Effect of Oxidising Agents.—For these experiments only dry and acetone yeast, or their water extracts were used, all containing either partly oxidisable or not autoxidisable components of cytochrome. It was found that H₂O₂, potassium ferricyanide, or potassium permanganate, added in small amounts rapidly and completely oxidise all the components of cytochrome without destroying them. On the addition of a reducer the four bands of reduced cytochrome reappear. While the salts of iron, manganese and cobalt have no effect on cytochrome, the cupric salts, for instance CuCl₂, in low concentration (M/1000) at pH 6 rapidly oxidise the reduced non-autoxidisable components of cytochrome. The addition of catechol oxidase with catechol to the suspension of dry or acetone yeast also oxidises cytochrome, which can be then reduced and reoxidised alternately.

Effect of Various Substrates.—Fresh bakers' yeast in 25 per cent. suspension, which in 2 c.c. reduces the oxidised cytochrome in 17 to 20 seconds, after half an hour's incubation with 3 per cent. glucose or fructose, reduces the pigment in 5 seconds. Incubation of this suspension for 5 hours, properly aerated, at 39° C. brings the rate of reduction of cytochrome back to 17 to 20 seconds. More interesting results are obtained with yeast suspension previously warmed to 52° C. in the usual way. In this suspension, according to the duration of

heating and the time the suspension was kept after heating, the reduction of cytochrome takes place in from 60 to 155 seconds, and this rate of reduction is not influenced by incubation with sugars. Such suspension was distributed in several tubes, 2 c.c. in each. One of the tubes, the control, received 0.2 c.c. of buffer, while each of the others received 0.2 c.c. of M/1 solutions of the following substances: sodium succinate, lactate, pyruvate and formate. The tubes were incubated for 1 hour at 37° C. and then cooled to 18° C., and the rate of cytochrome reduction in these tubes was rapidly compared. The results of this experiment are shown in Table IX.

Table IX.—Time of Reduction of Cytochrome in Seconds.

Exp. No.	Control.	Lactate.	Succinate.	Pyruvate.	Formate.	Formate + Lactate.
1	60	25	52	50	124	65
2	155	80	100	110	175	125
3	155	45	110	112	210	—
4	90	32	54	—	—	—
5	100	20	80	—	—	—
6	135	25	87	—	—	—
7	65	10	40	45	150	—

These experiments show that of all the substances added to yeast suspension warmed to 52° C., only lactate had a very marked effect in accelerating the reduction of oxidised cytochrome. The effect of sodium succinate and pyruvate is less marked, while formate, on the contrary, delays the reduction of the pigment.

E.—Oxidation and Reduction of Cytochrome in Heart Muscle Cells.

The thick stock suspension in phosphate buffer-mixture pH 7.3 of washed heart-muscle, prepared by the method previously described (p. 222), shows only faint bands of oxyhæmoglobin. These bands persist in the suspension standing for a long time, even in the cells which settle to the bottom of the tube, which shows that there is very little or no oxygen uptake in the preparation. The addition of KCN does not produce any visible change in the suspension. On the addition of a small amount of a reducer, such as $\text{Na}_2\text{S}_2\text{O}_4$, the faint bands of oxyhæmoglobin disappear completely and are replaced by the four strong bands of reduced cytochrome. On shaking the suspension with air, the four bands of reduced cytochrome disappear as the pigment is oxidised, and are replaced by the bands of oxyhæmoglobin. If, on the other hand, after reduction

with $\text{Na}_2\text{S}_2\text{O}_4$, M/1000 KCN is added to the suspension, which is then shaken with air, the band *b* disappears, while *a* and *c* remain unchanged. In other words, only the component *b'* of cytochrome can be oxidised in the presence of KCN. If to 2 c.c. of the suspension of muscle showing only the bands of oxyhæmoglobin, we add a drop of M/1 solution of sodium succinate, cytochrome begins to reduce, and in 15 to 20 seconds the bands *a* and *c* of reduced cytochrome are visible, although the bands of oxyhæmoglobin have not yet disappeared.

On shaking the suspension in air, cytochrome becomes rapidly reoxidised. In other words, in muscle suspension containing sodium succinate, cytochrome can be alternately oxidised (on shaking) and reduced (on standing) as in the suspension of living yeast cells.

A more striking effect is obtained when a drop of M/1 sodium succinate is added to 2 c.c. of muscle suspension containing a drop of 1 per cent. KCN. In this case the bands *a* and *c* of reduced cytochrome appear almost instantaneously, as if a reducer were suddenly added to the suspension, and later band *b* appears. On shaking this suspension with air, only component *b'* is oxidised, while *a'* and *c'* remain in a reduced state.

Of the other substances, lactate and fructose slowly reduce oxidised cytochrome. This is easily seen in the presence of a small amount of KCN, which prevents the reoxidation of components *a'* and *c'* once they have been reduced. It is important to add that the muscle suspension used in these experiments does not give a nitroprusside reaction; in other words, there are no SH groups which could act as reducers of cytochrome. Moreover, cystein added to the suspension of washed muscle reduces cytochrome very slowly, and on slight shaking it becomes completely reoxidised even in the presence of great excess of cystein.

X.—RESPIRATORY ENZYMES AND MECHANISM OF OXIDATION AND REDUCTION OF CYTOCHROME.

In the previous chapters we have established the main properties and the conditions affecting the activities of oxidases. We have determined also the properties of intracellular hæmatin compounds and the effect of various factors on the oxidation and reduction of cytochrome. Bringing together now these results and correlating them, we may draw the following conclusions as to the relationship between cytochrome and the respiratory enzymes of the cell.

(1) The cells of different organisms contain at least four distinct hæmatin

compounds : an unbound hæmatin, and the three hæmochromogen-like components, a' , b' and c' , of cytochrome. Of these four hæmatin compounds the free protohæmatin is autoxidisable, and when reduced combines with CO. The components of cytochrome differ, however, from typical hæmochromogens in two respects : the component b' , although autoxidisable in the presence of KCN (in washed dry yeast or in muscle cells) does not combine with CO. The components a' and c' of cytochrome are not autoxidisable and do not combine with CO. We know, on the other hand, that all the hæmochromogens prepared from any hæmatin and nitrogen compound are autoxidisable and combine with CO. We have seen, however, that at pH 13 the component c' becomes autoxidisable, and, in the presence of more alkali, the component c' of cytochrome even combines with CO, and so behaves as a typical hæmochromogen. The small amount of alkali used to make component c' autoxidisable could not possibly affect the iron-porphyrin portion of the molecule. Thus, the nitrogen compound which enters into the composition of a' and c' of cytochrome, seems to be of such a nature, or is combined with the iron-porphyrin in such a way, that it opposes the rearrangement of the molecule which takes place on the oxidation of hæmochromogen, or on its combination with CO. The hæmochromogen precursor of cytochrome and heliocorubin resemble component b' of cytochrome in being autoxidisable, and in not combining with CO.

(2) All the factors, such as KCN, ethyl cyanide, H_2S , CO, drying the cells, treating them with alcohol or acetone, etc., which inhibit the activity of indophenol oxidase, also inhibit the oxidation of cytochrome, and especially of its components a' and c' . The factors such as narcotics, or warming to $52^\circ C.$, which have very little effect on the activity of indophenol oxidase, do not interfere with the oxidation of cytochrome. This clearly shows that cytochrome in the living cells is oxidised by the indophenol oxidase.

(3) The factors such as washing the cells, warming them to $52^\circ C.$, lowering the temperature ($-2^\circ C.$) and the addition of narcotics, which inhibit the reducing systems of the cells that interfere with, or prevent, the indophenol reaction, also inhibit the reduction of cytochrome. In other words, cytochrome in the living cell is reduced by the same systems which inhibit the indophenol reaction of the cells, or which reduce the indophenol blue to indophenol white.

(4) The reducing mechanism can be considered here only in the sense of Wieland and Thunberg systems, namely, as dehydrogenating enzymes, activating the hydrogen of organic molecules, which thus become hydrogen donors. Cytochrome (or at least a' and c') which is oxidised by

indophenol oxidase acts as a hydrogen acceptor, or as a carrier between the two types of respiratory enzymes: dehydrases, or enzymes activating hydrogen, and oxidases, or enzymes activating oxygen.

To act as a hydrogen acceptor in biological activations, when the donator is an organic molecule activated by a dehydrase, is by no means a common property of all hæmatin compounds. On the contrary, a hæmatin, para-hæmatin or methæmoglobin, added to a thick suspension of washed muscle, containing sodium succinate and kept in nitrogen, remain oxidised for several hours, while cytochrome in such muscle suspensions, even when exposed to air, becomes reduced in a few seconds. Component *c'* of cytochrome which, in collaboration with R. Hill, was extracted from yeast in a more concentrated and pure form, giving a distinctly pink fluid with strong absorption bands, behaves in exactly the same way as the component *c'* within the cell. A drop of a strong solution of reduced *c'* added to a suspension of washed muscle immediately becomes oxidised, and, on the addition of sodium succinate to the suspension, the oxidised *c'* becomes reduced; while sodium succinate added to the solution of oxidised *c'* without muscle suspension, has no effect upon it.

This type of respiratory process, in which are involved the dehydrases, the intracellular hæmatin compounds, and the oxidases, can be schematically represented as shown in fig. 10*.

(5) The activity of this system depends therefore on a certain tension of oxygen, on the activity of oxidase, on the presence and distribution of suitable carriers (cytochrome and possibly other as yet unknown substances), on the activity of dehydrases, and on the presence of suitable molecules (metabolites) for activation. It is easy now to conceive conditions under which any one of these constituents may become a limiting factor in the respiratory process.

(6) The strength of the oxidase reaction given by untreated cells or tissue of various organisms shows only that the cells are not saturated with hydrogen donators. This may be due either to the low activity of the dehydrases, or to the low concentration of the molecules which are naturally activated in the cells. It shows also that the activity of indophenol oxidase is not the limiting factor in the respiratory process of such cells. On the other hand, a negative indophenol reaction does not denote the absence of the oxidase; on the con-

* This does not exclude other possibilities which have not yet been tested, such as (1) the existence of other non-hæmatin carriers, (2) the direct inter-reaction between an oxidase and hydrogen donators, and (3) the inter-reaction between hydrogen donators and molecular oxygen.

trary, the latter may be very powerful, but the cells, probably being saturated with activated molecules, so that the activity of the oxidase is diverted towards

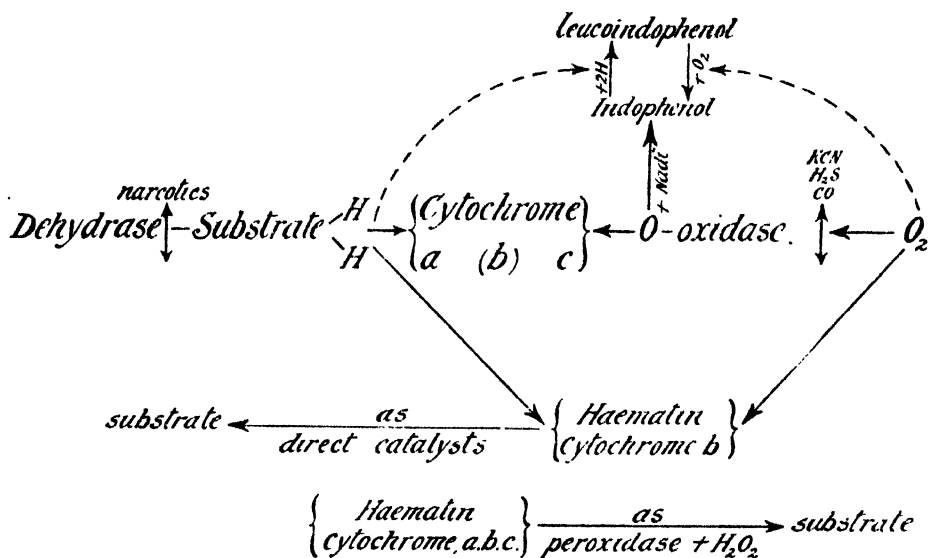


FIG. 10.—Diagram of the respiratory mechanism of the cell, showing the functional relationship between the intracellular haematin compounds and the respiratory enzymes such as dehydrases and oxidases.

the oxidation of these molecules and none of it is available for the oxidation of paraphenylenediamine.

The full activity of the indophenol oxidase towards paraphenylenediamine and other reagents can be observed only when the dehydrogenases are destroyed, inhibited, or desaturated, by starvation or washing the cells. The complete desaturation of cells by the elimination of all the metabolites can hardly be obtained in yeast cells without destroying the activating mechanisms. In muscle, on the contrary, the complete desaturation is easily obtained by washing the cells in water.

(7) KCN, H₂S and CO combine with some of the components of oxidase forming an inactive compound, with the result that cytochrome, or at least its components *a'* and *c'*, as well as paraphenylenediamine added to the cells, are not oxidised. The respiratory process can be still carried out through the medium of some autoxidisable carriers such as hæmochromogens, hæmatins, the component *b'* of cytochrome, or some as yet unknown autoxidisable substances. This residual respiration, according to the nature of the cell, may represent a larger or smaller fraction of the total respiration of the cell.

(8) The effect of narcotics such as urethane, which delay the reduction of oxidised cytochrome (a' and c'), and which bring out the positive indophenol reaction, is due to the fact that they have a much greater inhibitory effect on dehydrases than upon oxidases. We have seen that they do not interfere with the oxidation of paraphenylenediamine, which does not require activation, and that they do not affect the oxidation of cytochrome (a' and c').

(9) One of the effects of low or moderate temperatures on the respiratory process of the cell is due to the fact that the temperature coefficient of the activity of dehydrases is higher than that of oxidases. On lowering the temperature of the cells, a condition is reached when the velocity of hydrogen activation becomes much slower than that of the activation of oxygen. Then the reduction of oxidised cytochrome is delayed and the indophenol reaction becomes positive. The difference in the temperature coefficient of these two systems explains also why a rise of temperature distinctly increases the inhibitory effect of CO on the oxygen uptake by yeast cells (Warburg), while it has no effect, or the opposite effect upon CO-inhibition of oxidation of paraphenylenediamine or of catechol.

The CO, as was shown by Warburg, inhibits the O_2 uptake of yeast only when the latter is incubated in some nutrient medium; in other words, when the cells are more or less saturated with the combustible material. It seems, however, that saturation of the cells with nutrient molecules does not affect the oxidase-cytochrome system, because the latter will not react with these molecules until they are activated by dehydrases. As, on the other hand, the temperature coefficient of dehydrase activity is higher than that of oxidase activity, with rise of temperature the cell can be brought nearer the state of saturation with activated molecules, so that the degree of inhibition of oxidase by CO will increase, although the affinity of oxidase for CO may actually diminish.

This shows that in Warburg's experiments (1926) the effect of higher temperature on the value of $K = \frac{n}{1-n} \cdot \frac{CO}{O_2}$ was not due to the corresponding increase in the affinity between the oxidase and CO, but to the more complete saturation of the cell with the activated molecules.

Warming the yeast suspension to $52^\circ C.$, as was often done during our experiments, has undoubtedly two effects: (1) it rapidly desaturates the cells from substances which are usually activated; and (2) it destroys irreversibly some of the activating mechanisms. It diminishes, however, to a much greater degree the activity of dehydrogenases than that of oxidases, with the result

that the reduction of oxidised cytochrome is greatly delayed and the oxidase becomes available for oxidation of paraphenylenediamine.

Warming the tissues to higher temperatures (70° C. and above) destroys both dehydrases and oxidases, and even decomposes components *a'* and *b'* of cytochrome.

(10) Drying the cells or treating them with acetone has a much more destructive effect upon oxidases than upon some, at least, of the dehydrases, which still remain very active. Cytochrome in a suspension of such cells is in a reduced state, and, on shaking, it oxidises only partly or not at all, while the indophenol reaction is very feeble or negative. The oxygen uptake of acetone yeast suspended in buffer or in nutrient media is very small, representing, according to Meyerhof, not more than 2 per cent. of the oxygen uptake of normal yeast. As the oxidase in this preparation is almost completely destroyed, this residual respiratory process is carried out only by dehydrases and some of the autoxidisable hydrogen acceptors. This explains the interesting results obtained by Meyerhof (1918) with the respiration of acetone yeast, namely, that it is still inhibited by narcotics, very little or not at all inhibited by KCN, and accelerated by methylene blue which acts as an additional, and more efficient, autoxidisable hydrogen acceptor.

(11) The organic molecules which are activated by dehydrases can be removed from the cells either through starvation or by washing, with the result that the reduction of cytochrome is inhibited or abolished, and the oxidase becomes available for the oxidation of paraphenylenediamine. The addition of various substances, such as sodium lactate, to yeast (warmed at 52° C.) or succinate to washed muscle accelerates the reduction of cytochrome but retards the indophenol reaction.

(12) The oxidation of sodium succinate by washed muscle can be taken as an example of a respiratory process of the type represented in fig. 9. We have seen, in fact, that cytochrome oxidised by indophenol oxidase, is reduced by sodium succinate added to the muscle, while this salt has no effect on the oxidised component *c'* extracted from the tissue. That the oxidation of sodium succinate in muscles is carried out by two activating mechanisms: a succindehydrase of Thunberg and an oxidase, was already demonstrated by Fleisch (1924) and by Szent-Györgyi (1924). It was shown, however, by Dixon (1927) that, although the oxidation of sodium succinate is inhibited by KCN, it is not inhibited by CO, which as we have seen inhibits the indophenol oxidase. The results obtained by Dixon can be explained in two different ways: either (1) that the oxidase involved in this process is different from indophenolase; or,

(2) what is more probable, that the oxidase is the indophenolase, but, being in a much greater concentration than dehydrase, was not saturated with hydrogen donators. Consequently, the partial inhibition of the oxidase by CO could not be detected from the oxygen uptake of the preparation. In fact, the succindehydrase preparation of Dixon was found to be rich in indophenol oxidase.

XI.—SOME OTHER FUNCTIONS OF INTRACELLULAR HÆMATIN COMPOUNDS.

We have seen that the three components of cytochrome can act as hydrogen acceptors, or as carriers between the two activating mechanisms of the cell; the dehydrases and the oxidases. In addition to this, the intracellular hæmatin compounds may also have other functions in the cellular respiration.

(1) The autoxidisable compounds such as the component *b'* of cytochrome, the hæmochromogen precursor of cytochrome and the unbound intracellular hæmatin may also act as carriers between the hydrogen donators and molecular oxygen. These compounds, especially the protohæmatin, are not so easily reduced as the components *a'* and *c'* of cytochrome, and it is quite possible that they act mainly as hydrogen acceptors in other systems to which they are more specifically accessible and which have a higher reduction potential. This property of autoxidisable hæmatin compounds may partly account for the residual oxygen uptake by the cells poisoned with KCN.

(2) Hæmatin compounds, as was shown by Robinson (1924), act as efficient catalysts in the autoxidation of linseed oil. Harrison (1924) found that hæmatin is also capable of catalysing the oxidation of cystein and of glutathione, and, for cystein, this was recently confirmed by Krebs (1928). This shows, therefore, that the intracellular hæmatin compounds, especially the autoxidisable compounds such as *b'* of cytochrome, the hæmochromogen precursor of cytochrome and the protohæmatin, in addition to their function as carriers, may act also as direct catalysts in the oxidation of substances not activated by specific dehydrases.

(3) The four intracellular hæmatin compounds, as was previously shown, have also the properties of thermostable peroxidases capable of promoting the oxidation of various substances by means of hydrogen peroxide. It was shown, on the other hand, that hydrogen peroxide may be formed as the result of the activity of Schardinger's, or xanthine oxidase systems, as well as in the oxidation of thiol groups (Dixon, 1925; Harrison and Thurlow, 1926). If, therefore, the formation of hydrogen peroxide within the cell could be proved to be of general occurrence, the hæmatin compounds acting as per-

oxidases could then be considered as capable of inducing a series of secondary oxidations.

XII.—THE NATURE OF THE OXIDASE.

The respiratory enzymes which take part in cellular respiration and which have been considered in this paper belong to two distinct types: (1) the dehydrases which activate the hydrogen of organic molecules (metabolites); and (2) the oxidases which activate molecular oxygen. All the foregoing experiments clearly demonstrate that it is inconceivable to ascribe the whole of the respiratory process to the activity of only one type of enzyme to the complete exclusion of the other type.

Consideration of the nature of dehydrases, their specificity and the mechanism of their action, which form the subject of important investigations by Wieland, Thunberg, Dixon, Quastel and others, lie outside the scope of this paper. For the discussion of this subject the reader is referred to the Presidential Address delivered by Sir Frederick Hopkins to the Physiological Congress at Stockholm in 1926.

The literature dealing with oxidase systems of the cell is very extensive (see Kastle, 1909; Batelli and Stern, 1912; and Oppenheimer, 1926), but our knowledge as to the nature of these ferments, and especially the mechanism of their activity is still very incomplete. The rôle of metals such as manganese in an oxidase system of the type of laccase was shown already by Bertrand (1897). According to Spitzer (1898) the indophenol oxidase of animals is an Fe-nucleoproteid compound, where iron plays the main rôle in oxidation. He ascribed to oxidases a very important part in the respiratory processes of organisms. The rôle of iron in the oxidase of plants (*Schinus*) was emphasised by Sarthou (1900). Other observers, however, claimed to have obtained oxidase preparations without this metal. The effect of KCN, and later of H_2S , on the activity of oxidases was known to many observers since Schönbein (1863b), but the significance of this poisoning effect was misunderstood for a long time.

The view that iron plays an important part in biological oxidations was energetically defended by Warburg, who supported his view by a series of experiments on the respiration of sea-urchins' eggs, and on that of avian red blood corpuscles; and by his "respiratory model" experiments consisting in the oxidation *in vitro* of certain amino-acids by the iron of charcoal surfaces. He has also put forward the view that the inhibition of the respiratory process with KCN is due to its combination with the catalytic iron, which thus becomes

inactivated. The amount of KCN which is necessary to abolish the oxygen uptake of cells he found to be approximately in stoichiometric relationship with the amount of iron present. He believed also in the existence of a quantitative relationship between the amount of iron present in the cells and their respiratory activity (1914-1924, 1925).

At that time Warburg was unaware of the presence, in addition to the unmasked iron compounds, of iron in the form of widely distributed intracellular hæmatin compounds such as cytochrome and free hæmatin. Following the recent developments of our knowledge of these compounds, Warburg (1926) has tested the effect of carbon monoxide on respiration, and found that at a high partial pressure it depresses the oxygen uptake of cells. The study of the effect of light of definite wave-lengths on the respiration of cells poisoned with carbon monoxide, brought Warburg (1928b) to the conclusion that the "respiratory ferment," which as we have shown is the indophenol oxidase, is also a hæmatin compound and represents only a very small fraction of the total cellular hæmatin.*

In this paper, comparing the respiratory ferment with cytochrome, he states: "Just as hæmoglobin is a degenerated ferment because its iron atom is catalytically inactive, so is cytochrome a degenerated ferment because its iron atom is incapable of reacting with oxygen." This statement seems to imply that the main properties of the "respiratory ferment" are found in a hæmatin compound which reacts with O_2 . I am certain, however, that the indophenol oxidase is not at all identical with the autoxidisable intracellular hæmatins, as the properties of this oxidase differ markedly from those of all the known hæmatin compounds. Several important questions still remain to be answered before we consider the view of the hæmatin nature of oxidase as established. Of these questions the following are the most important: -

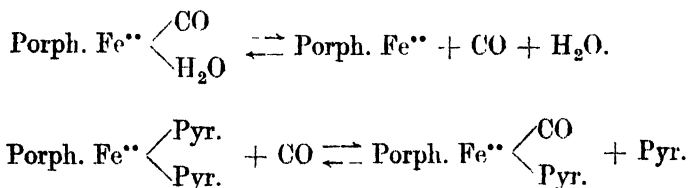
(1) One of the most important properties of the oxidase (or Warburg's respiratory ferment) is the inhibition of its activity by a very small amount of KCN. The addition of a very small amount of KCN to a reduced hæmatin or hæmochromogen has, however, no effect on their property of oxidation. In higher concentration KCN combines with reduced hæmatin giving KCN-hæmochromogen, which is one of the most easily autoxidisable hæmochromogens. If, therefore, KCN inhibits the catalytic properties of a hæmatin or of a hæmochromogen (Krebs, 1928), it shows that either this property is independent of their ability to react with molecular oxygen, or that the catalytic oxidations

* It is important to note that according to Euler, Fink and Hellström (1927) the hæmatin iron of cells of yeast represents $\frac{1}{10}$ to $\frac{1}{20}$ of the total iron of these cells.

taking place in the presence of hæmatin compounds are due to some products of their decomposition which are spectroscopically invisible and are inactivated by KCN. Finally, it is possible that KCN influences this reaction in some other way which still remains to be determined.

(2) H_2S , which like KCN has a very great inhibitory effect upon the activity of oxidases, has no effect on the oxidation of the hæmatin or hæmochromogen compounds. It does not combine with them, and in high concentration acts as a temporary reducer, but never as a specific poison.

(3) CO and O_2 seem to compete for the oxidase, but there is no evidence of such competition for a catalytically active hæmatin compound. Oxidised hæmatin, parahæmatin or methæmoglobin, even when kept in pure CO, do not combine with this gas; while the reduced (ferrous) compounds of hæmatin combine with CO at very low tensions of this gas. The equilibrium between the reduced hæmatin or pyridine hæmochromogen and CO, as was shown by Hill (1926) is of the following nature :—



In other words, in the case of CO-pyridine-hæmochromogen the CO competes, not with O_2 , but with pyridine.

(4) The only case where there is a definite equilibrium between hæmatin, CO and O_2 is represented by hæmoglobin, which does not undergo oxidation but oxygenation, and the iron of which is catalytically inactive (Warburg, 1928).

(5) From the effect of light of different wave-lengths on the reactivation of the respiratory process of yeast cells poisoned with CO, Warburg was able to plot a photochemical absorption spectrum resembling that of hæmatin, and this brought him to the conclusion that the respiratory ferment is also a hæmatin compound. Such experiments would naturally prove this assumption if they could be carried out with ferment separated from the rest of the cells. Unfortunately, the effect of light can be studied only on cells already containing four other hæmatin compounds. We cannot, therefore, dismiss the possibility that light may have acted here on some of these hæmatin compounds by promoting their activity in a way which could affect the equilibrium between oxidase, CO and O_2 .

(6) Moreover, the polyphenol oxidase extracted from potato, which shares

with the indophenol oxidase the properties of being inhibited with KCN, H_2S and CO, when poisoned by the latter does not seem to be reactivated by light.

(7) All the hæmatin compounds show a distinct peroxidase property, while the concentrated and very powerful polyphenol oxidase of potato, when properly purified, does not give a peroxidase reaction.

(8) The autoxidisable hæmatin compounds, on the other hand, can oxidise neither artificial chromogens nor the reduced components a' and c' of cytochrome which are so easily oxidised by indophenol oxidase.

It may be possible that the cells either contain several distinct oxidases having different properties or that the enzyme which we call indophenol oxidase does not correspond to one substance but comprises two components differently affected by the reagents such as KCN and CO. That one of these components may have a hæmatin nucleus is quite conceivable, since we have shown that the cells of all organisms are capable of synthesising several hæmatin compounds taking an active part in cellular respiration.

SUMMARY.

(1) Cells of aerobic organisms contain a widely distributed respiratory pigment, cytochrome, which is composed of three hæmatin compounds (a' , b' and c'); and an unbound hæmatin compound similar to the protohæmatin of hæmoglobin.

(2) Of these four hæmatin compounds, the components a' and c' of cytochrome are not autoxidisable, while b' of cytochrome (in washed muscle and dry yeast cells) and the unbound hæmatin are autoxidisable, and the latter in the reduced state combines with carbon monoxide.

(3) The hæmochromogen precursor of cytochrome is also autoxidisable, but does not combine with CO.

(4) The hæmatin compounds of the cell are responsible for the thermostable peroxidase reaction, which is shown by the oxidation of various chromogens, such as benzidine, guaiacum, and paraphenylenediamine, in the presence of H_2O_2 .

(5) The cells of yeast, muscle and other tissues also contain an insoluble thermolabile true indophenol oxidase, which catalyses the oxidation of paraphenylenediamine or of the "Nadi" reagent.

(6) The activity of this oxidase is inhibited by very small concentrations of KCN and H_2S , and by CO at high partial pressure and in the dark. The inactive CO-oxidase compound is dissociated when the cells are exposed to

light, liberating the active oxidase. The latter is greatly damaged in cells dried in air, or treated with acetone or alcohol, and is completely destroyed in cells warmed to 70° C.

(7) All the factors which inhibit the activity of oxidase, or destroy it completely, affect in the same way the oxygen uptake of the cells, which shows that the indophenol oxidase takes an important part in cellular respiration.

(8) The polyphenol or catechol oxidase of potato, which can be obtained in a clear solution, is also inhibited by KCN, H₂S and CO and destroyed on warming to 70° C. This oxidase is not damaged by alcohol, or drying, and light has no effect on the CO inhibition of its activity.

(9) The indophenol oxidase is responsible for the oxidation of cytochrome, especially of its non-autoxidisable components *a'* and *c'*, as the oxidation of cytochrome is inhibited or abolished by the same factors which inhibit or abolish the activity of the indophenol oxidase.

(10) Cytochrome in the living cells is reduced by various organic molecules (metabolites), which being activated by dehydrases become hydrogen donors. All factors which inhibit the activity of the dehydrase system of the cell, such as narcotics, warming to 52° C., and very low temperature, also delay the reduction of oxidised cytochrome.

(11) Cytochrome acts therefore as a carrier between two types of activating mechanisms of the cell: (1) the dehydrases activating the hydrogen of organic molecules; and (2) the indophenol oxidase activating oxygen. Cytochrome thus acts as a hydrogen acceptor which is specifically oxidised by the indophenol oxidase.

(12) The autoxidisable hæmatin compounds, *b'* of cytochrome, unbound hæmatin and the hæmochromogen precursor of cytochrome, may also act as carriers between the hydrogen donors and the molecular oxygen, and also as direct catalysts promoting the oxidation of substances which are not activated by specific dehydrases.

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On the Nature of Postural Reflexes.

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The "reflex standing" seen characteristically in the spinal animal, and exaggerated in the decerebrate animal, when analysed myographically is found to be a reflex contraction which is caused by stretch of the muscle itself, the "stretch reflex" (31). In upright postures gravity provides the stretch which elicits the reflex, and the resulting antigravity stretch response is prominent in all limb extensors. This response is a typical extensor reflex and is found only in fractional form in the flexors (5) in the cat, dog, and other quadrupedal mammals. In such animals as the sloth where the typical postures involve stretch of flexor muscles, decerebrate rigidity involves the flexors (46).

Besides its peculiar predilection for extensor muscles the stretch reflex would appear to be endowed with some property which renders it relatively resistant to fatigue, both central and peripheral, compared with other types of reflex innervation. A number of explanations have been put forward to account for the relative unfatiguability of the postural reflexes, and these may be classified in three categories, commencing with those of Mosso (38), Bottazzi (10), de Boer (9) and Langelaan (30), that muscular rigidity is secured by means of a special form of contraction in striped-muscle fibres, secured by a slow contraction process, or else increased plasticity, in the sarcoplasm. The sympathetic nervous system is thought to control this process by Mosso, de Boer, and Langelaan, while recently Frank (21) and Ranson (44) suggest that the dorsal roots control the mechanism. Secondly there is the theory of Hunter (29), who suggests that rigidity is the special function of a particular type of muscle fibre. Finally there is the suggestion of Forbes (19) that the rigidity was produced by muscle fibres activated by a motor discharge of high rate, throwing them into conventional motor tetanus. He suggested that fatigue was lessened by alternate activity and rest in different fibre groups—a rotation of activity.

An examination of the reflex mechanism has been made, the stretch reflex being used as the basis for observations. The methods and technique used for investigation are those which have been described in detail in the last paper on this subject (15).

1. -The Reaction of Resting Muscle to Passive Stretch.

Since the stretch reflex depends upon the effect of changes of tension, both internal and external, upon the muscle fibre, it is essential first to investigate the effect of stretch upon inactive, resting muscle. The method is the same as for investigating stretch reflexes, namely, recording the tension to which the muscle attachments are subjected for different lengths of muscle fibre, and

during linear change from length to length. This was done as a control to their experiments by Liddell and Sherrington (31), and they described the typical curve of tension of a paralysed muscle which is extended from one length to another. This curve was concave upwards and rose more steeply as the muscle approached the greater length. They also described a preliminary short rise in tension with the onset of the stretch. This curve has been reinvestigated with a delicate isometric myograph of the mirror type (14a) (52) which registered small tension changes through a range which was not likely to impose a strain on the endomysial tissues.

A typical parallel fibred flexor (semitendinosus) gives a curve which is illustrated in text-fig. 1, A. The increase in stretch is linear, but the corresponding rise in tension shows a large preliminary acceleration with a later plateau, and thereafter rises along a curve which is concave upwards until the stretch ceases. The curve is similar to that obtained by Liddell and Sherrington in extensor muscles, except that the preliminary "hump" is proportionally greater and indicates that at the first stretch from the resting level the muscle behaves as a rigid structure, maintaining its length in spite of increased tension pull. The myograph gives, while the muscle maintains its length. This phase is followed at a certain tension (31 gm. in this case) by a phase of relatively constant tension, the plateau, during linear increase of length. Following this the muscle enters a third phase, which is a curvilinear increase in tension during the continued linear increase in length. A rubber band subjected to the same stretch records a linear increase in tension, which is a relative index of the increase in length (text-fig. 1, E). An inextensible band depresses the myograph directly and the tracing would rise very steeply along a straight line. The three phases may be summarised by saying that a muscle, upon being subjected to a linear increase in stretch, at first behaves as an inelastic body and later as an elastic body.

This phenomenon, the "preliminary rigidity," is a characteristic of both flexor and extensor muscles, and is found whenever the muscle has rested for more than a very short period at any length. The minimum interval (less than 1/10 second) for its appearance has not been determined exactly, owing to the technical difficulties involved.

When the stretch is released at even speed the reverse sequence of events occurs (text-fig. 1, B). The muscle again behaves as a rigid body and the tension falls steeply, as if the muscle tended to retain its length. Having fallen a certain amount, however, a plateau appears, and the tension remains thereafter declining along a curved line. The muscle behaves as an inelastic

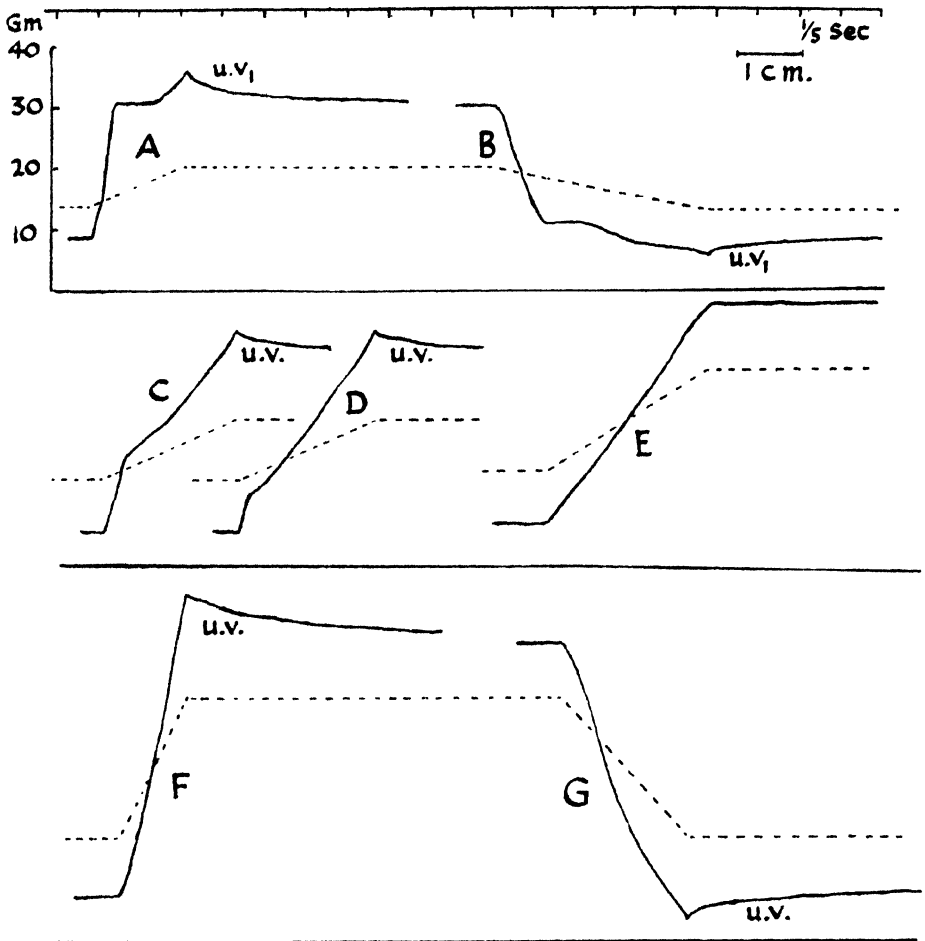


FIG. 1.—Curves of tension in response to stretch and stretch release. The dotted line in each case shows the level of the table to which one end of the material is fixed, the continuous line is the record of the myograph image recording the tension. A and B—Stretch and stretch release (4.5 mm.) of *M. semitendinosus*, cat. C and D—Two stretches repeated with an interval of 3 seconds. E—The stretch tension of a rubber band. F and G—Stretch and stretch release (1 mm.) of piece (13.5 mm.) of tendon of *M. semitendinosus*. The tension and time scales with curve A apply to all. The curves in this and subsequent text-figs. are tracings from photographic records, and all read from left to right.

body, later transformed into an elastic one, both in release of stretch and in onset of stretch.

When a new length is reached and maintained without further stretch a reaction typical of a viscous component is manifested as a small decreasing

decline in tension, beginning the moment the length is maintained constant at the greater tensions, and a progressively smaller rise in tension occurring immediately the length is maintained constant at the lower tensions (*v.r.* in text-fig. 1, A, B, C, D). Since the muscle has had the perimysium removed as much as possible without damaging the blood supply, the only elements which remain for consideration are the endomysium, which in this muscle is negligible in quantity, the contractile muscular element, and the tendon and tendon aponeuroses. A length of tendon responds with a typical "viscoid" reaction (text-fig. 1, F, G), even to the recovery of tension upon cessation of shortening, but no "stationary rigidity" is found in it.

"Stationary rigidity," which causes the curve to depart from the line of an elastic body, is therefore assumed to be a peculiarity of muscle substance and a property of the muscle fibre. It lessens in extent with each stretch upon rapid repetition of the stretch (text-fig. 1, C, D) but recovers with rest at a stationary length.

The "viscoid reaction," or readjustment of tension which takes place continuously during and after the stretch, is represented in the muscle stress-strain diagram of Hartree and Hill (27) and is here considered to be the same phenomenon as the plasticity of Langelaan (30) viewed from the point of view of length rather than that of tension. Langelaan has claimed an effect of the sympathetic nervous system on muscle plasticity in frog muscle, and it is important therefore in considering the stretch reflex to know if the stationary rigidity or the viscous reaction or both are affected by, or are a product of, any nervous mechanism. The tracings in text-fig. 1, A, B, C and D are from a muscle with all nerve supply intact but no stretch reflex present. As the illustrations of Liddell and Sherrington (31) reveal, it is present in the extensors with all innervation intact, but under complete inhibition, and is the same after section of the motor nerve. The presence or absence of the sympathetic connections, or excision of the sympathetic ganglia in successive preparations, do not in my experience exert on either reaction any appreciable effect. The absence of the ventral root supply, leaving the dorsal root and sympathetic supply intact, also does not affect either reaction.

The viscoid reaction (which is regarded as non-specific for muscle) and the stationary rigidity (a plastic reaction of the muscle-fibre) appear unaffected, therefore, in all circumstances of resting muscle. It does not appear likely therefore that there is in mammalian muscle any nervous plastic adjustment which is independent of the function of the ventral root innervation. Both reactions presumably affect the contractile process, but the viscoid reaction

has no especial relation to the contractile mechanism and is insignificant in size in relation to the mechanical reactions of that mechanism.

Muscle in a state of pure motor (*i.e.*, non-reflex) contraction, as Gasser and Hill (25) showed, reacts to rapid stretch and stretch release as a totally rigid body, as if the stationary rigidity were then complete. A muscle exhibiting the stretch reflex behaves to very rapid stretches and releases also as a rigid body, but to slower stretches and releases as an elastic one, as may be seen in the curves of Liddell and Sherrington (31). According to the hypotheses of dual nature of muscle tonus this may be evidence of a special ill-defined plastic change, commingled with the reactions of muscle exhibiting the conventional contractile process (motor twitch and tetanus).

The curves of the stretch reflex, when obtained from a muscle whose stretch is inseparable from an increase of the conventional contractile element (the latter evidenced by the presence of the de Barenne-Buytendijk action-current rhythm) and whose release is inseparable from a decrease in that element, obviously cannot be entirely due to plastic change. The size of the action-currents suggests but a small amount of contraction process, but this criterion will be subjected in the following sections to a close investigation.

2.--The Mechanical Muscular Effect of the Stretch Reflex.

(a) *Data Derived from a Study of Contraction Duration.*—In the nerve-muscle preparation, excitation by break-shock causes a rise in tension in the muscle which outlasts the stimulus by a period, part of which is called the “after-action” of the twitch or tetanus (23). In the case of a twitch this is seen as the interval between the conclusion of the action current and the occurrence of the angle of commencing relaxation (22), as well as the interval occupied by relaxation. In the case of a motor tetanus the interval between the end of the last action-current and the angle of commencing decline from the plateau is the plateau after-action. In the slow red muscles of the ankle and knee joints (soleus and crureus) it is the length of this period of after-action which is the most obvious feature of their slowness, forming the most obvious difference between them and the rapid muscles. This long after-action of slow muscle carries with it some interesting features, two of which are of importance in the present analysis. These are (i) a prolongation of this after-action in the course of summation, and (ii) the evidence which this enhancement gives of the degree of homogeneity of the duration of the muscle elements.

Fulton (24) in his analysis of summation in frog gastrocnemius noted that within the first 38–40 σ after a first break-shock excitation, a second break-shock, if effective, caused a mechanical response, of which the after-action was longer, and the tension greater, than if it had been alone. In other words summation within 40 σ was summation in the duration of the second twitch as well as in its tension development. Myographic analysis with isometric technique reveals often an astonishing summation in duration in mammalian red muscle. For instance, in a typical soleus of the cat the contraction duration, measured from action-current to angle of the second twitch response for various intervals between the two stimuli, varied from 245 σ , at 3.6 σ from the first excitation when it was earliest obtainable, to 300 σ at 4.4 σ , 5.1 σ and 6.0 σ after the first excitation, and dropping to 295 σ when the two stimuli were 13 σ apart. Alone, each stimulus gave a twitch response of 200 σ duration. In the earliest summation in this muscle, therefore, there is a large augmentation of after-action, which gradually lessens as the stimuli are more spaced (text-fig. 2, B).

Furthermore, a series of stimuli at a rapid rate (above some 35 a second) can augment the duration of the after-action even more than two single stimuli, and this serial summation bears a curious relationship to the number of shocks at the rapid rate. Thus in one case (text-fig. 2, A) a single break-shock produced a plateau after-action of 210 σ (interval between onset of last action current and angle of commencing relaxation), while 2 shocks at 54 a second (18.5 σ apart) produced an after-action of 320 σ , 5 shocks at this rate 360 σ , 10 shocks 340 σ , and 18 shocks 260 σ ; but 36 shocks, which carried the muscle to the plateau, gave only 220 σ after-action. After any longer period of tetanic stimulation the after-action reaches a constant value, which is a little less than the twitch duration, until fatigue sets in and augmentation again occurs. This relationship of after-action to the duration of tetanic stimulation at this rate is shown with superimposed traced records in text-fig. 2, A, from this soleus.

The degree of summation of the duration of the after-action is generally found to vary directly with the twitch duration characteristic of the muscle in the preparation examined, though this is not always so. Some degree is usually found in the slow soleus muscle of the cat, but much less so in rapid muscles according to their duration. No summation of duration, even at minimal interval, is found in a muscle such as a gastrocnemius or flexor, if these muscles have differentiated full rapidity in the preparation examined. Varying inversely with this influence of the first response on the duration of the

second is the effect of the first response on the tension height of the second. In a rapid muscle this tension effect dominates the summation, and the duration of the second response may even suffer impairment while its added tension

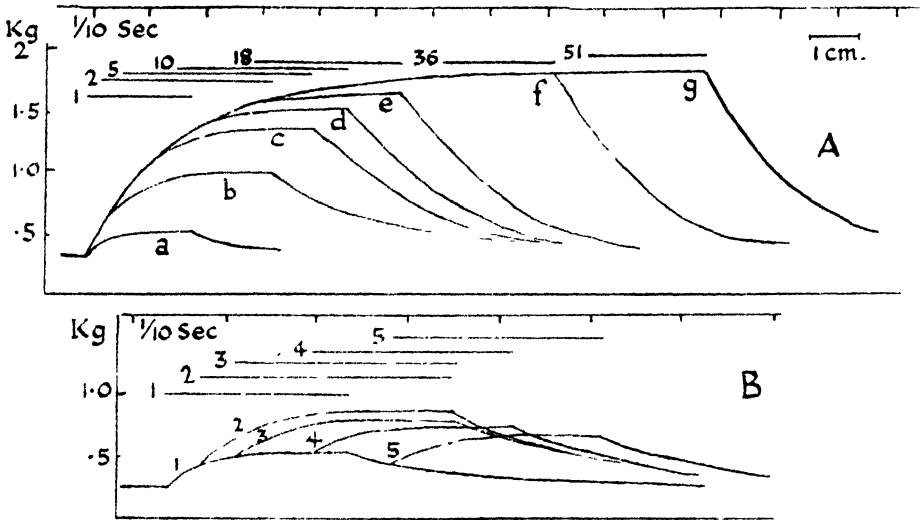


FIG. 2.—Superimposed isometric records of motor contractions of *M. soleus* (cat). A—A stimulus to the motor nerve, of a regular series of break-shocks at 54 a second, is allowed to continue for periods of varying length. (a) for 1 break-shock, (b) for 2 break-shocks, (c) for 5 break-shocks, (d) for 10 break-shocks, (e) for 18 break-shocks, (f) for 36 break-shocks, and (g) for 51 break-shocks. Plotted above each contraction is a line which shows the time elapsing between the termination of the last action current and the angle of commencing mechanical relaxation in each case. This line represents the duration of after-action for each. B—The tension record of a single motor twitch has superimposed on it the curves produced by a second break-shock occurring after varying intervals from the first. The duration of contraction of the twitch and of each second response is plotted above.

is very much greater than it is alone. These variations in duration of after-action lie quite beyond any possible result of admixture of fibres of different durations of response in soleus, both because they occur in maximal responses and because it has been found by experimental mixing of these muscles that it is possible to detect a rapid element in a slow response in less proportion than 5 per cent. by plateau evenness.

High importance attaches in the present investigation to the character of the after-action as an indication of the type of excitation which has preceded it. (i) If the plateau after-action is longer than the after-action of a twitch of the same muscle at the same length, then that after-action has been preceded

by two or more stimuli within a period corresponding to the duration of the muscle. (ii) If the after-action duration is markedly augmented (over 50 per cent.), then a short tetanus of high rate (over 35 a second) has occurred; and if less summed, then two stimuli at any interval up to that of the duration of the muscle. (iii) If the after-action is equal to the duration of twitch contraction, then the discharge must have been either a single excitation, or a series of single excitations separated by intervals corresponding with, or greater than, the duration of a single twitch. (iv) If the plateau after-action be less than the duration of the twitch then a fused tetanus of more than 20 a second has occurred and reached its plateau.

The second phase of after-action, the relaxation, varies with the duration of the stimulation period; this can be seen in text-fig. 2, A, the relaxation being the **more** rapid when duration-summation is minimal. Once the plateau is reached and the preliminary augmentation of duration has given way to a slight decrease in after-action duration, the curve of relaxation settles to a relatively constant value. Between the relaxation from a tetanus at a rapid rate of stimulus and that from a slow rate of stimulus when each has reached the plateau the difference is one of greater rapidity of fall for the more rapid rate. It is found that for maximal responses the relaxation from the plateau at a slow rate is exactly the same as the relaxation curve from a short tetanus of rapid rate reaching that plateau height. The relaxation from a given tension level therefore allows a distinction between a group with rapid, more concave fall (fibres innervated at 35 a second or over, long enough to reach their maximum) and a group with a delayed fall (fibres innervated at 35 a second or over for a short period, or fibres innervated at a much slower rate for a long period). These two groups with delayed fall can be distinguished by the plateau after-action, for although their curves are the same, that of the former group occurs much earlier after cessation than that of the latter group. The amount of augmentation of after-action duration varies from animal to animal, and it is necessary to determine the twitch-duration value, tetanus augmentation, and relaxations, in each case when comparison is made with the reflex.

Furthermore, since the after-action and its augmentation are increased by fall of temperature and vary with length of fibre, it is essential in comparing one response with another to keep the muscle at exactly the same length and to allow a minimum of time to elapse between them. The required data for comparison of motor contraction and reflex were therefore obtained in rapid series, the motor contractions being recorded before, and between, reflex

contractions by stimulating the intact muscle nerve during stimulation of the same afferent which was used to obtain the maximum inhibition of the reflex. Such motor responses during inhibition were not found to differ in any way from the response from the muscle obtained by stimulation of the distal portion of the muscle nerve within two minutes after its section proximally.

(b) *The Tendon Jerk.*—It is obvious that, in the tendon jerk for instance, soleus will give an index, whether one impulse, or more than one impulse, has arrived in the majority of the fibres, for as explained previously (15) the shape of the short action-current of the tendon jerk is explicable either as a brief tetanus (6), or else as a number of single waves arriving out of time in different fibres. A tendon jerk in a soleus of a decerebrate animal and a twitch caused by break-shock to the intact motor nerve, under circumstances apart from reflex inhibition exactly similar, are generally of the same duration; this indicates that the action-current of the tendon jerk, although longer than that of the twitch, is only a compound of single action-currents in different fibres slightly scattered in time. A slight difference (of some 5–10 σ) does occasionally occur, especially with small jerks, but this is never as large as the augmentation by two stimuli with short interval in the same circumstances; it is due probably to the varying shortening of the contractions of different height.

By the all-or-none hypothesis (Gotch (26), Lucas (35), Adrian (1) and Pratt (43)) a single impulse arriving at any resting fibre exerts the same effect under the same conditions. From this the tension height of the mechanical twitch compared with tension height of the tendon jerk indicates that the latter involves only a fraction of the muscle fibres, or else that the response of the jerk is impaired by some phenomenon of the sympathetic or dorsal root system. That the response is not so "impaired" can be demonstrated by removal of the lumbar sympathetic chain, and in observations on quadriceps after removal of the sympathetic ganglia supplying that muscle, and by the ability with which the tendon jerk may be exactly imitated by means of stimulating by break-shock one ventral root supplying the muscle.

The jerk just examined is one which is unaccompanied by any other reflex effect. A higher initial tension gives a slight degree of stretch reflex as background, and the jerk then shows a "hump" or myotatic appendage (6, 15) checking its course of relaxation. A break-shock to the intact muscle nerve under these circumstances, without inhibition to damp central effects, produces a motor twitch of the same duration, also with a "hump." This twitch, if not smothered by a heavy background of stretch reflex is exactly similar to that produced under complete inhibition, and therefore it is presumed that

here the ascending volley in the motor fibres has left the centre refractory (15) for the ascending afferent excitatory and inhibitory volley set up in the afferent fibres by the same stimulus. The "hump" or myotatic appendage is unaffected by removal of the sympathetic ganglia supplying the muscle. In a recent paper (15) it was shown to be the myotatic recovery or rebound from a proprioceptive inhibition accompanying the tendon jerk.

The tendon jerk in soleus after spinal section is also of duration similar to that of a muscle twitch in the same circumstances. Occasionally a difference of the order of 5–10 σ is encountered, but it is clear from the silent period following the action-current of the jerk (15) that if more impulses reach the fibres after the first these must occur within 10 σ of the first impulse, and this would cause a much greater augmentation of duration in these preparations.

The tendon jerk, which is a fractional manifestation of the stretch reflex, differs from a maximal motor twitch only in tension development (fewer muscle fibres involved) and in slight asynchronism. Each motor nerve fibre conveys but one impulse before the "appendage" occurs.

(c) *The Stretch Reflex*.—Soleus is a muscle which, for a reason to be explained later, responds well in the stretch reflex. It is possible to inhibit this reflex in the muscle completely and instantaneously, by a strong repetitive stimulus to an afferent nerve, preferably the hamstring nerve of the same side. The completeness and suddenness of the inhibition can be gauged by leading the action-currents from two parts of the muscle to two galvanometer strings. The action-current stream is then found to cease simultaneously in all parts of the muscle. In such circumstances, the motor discharge being stopped suddenly in the whole muscle, the resulting mechanical relaxation can be examined for features which bear on the state of excitation immediately preceding (*v.s.* § 2 (a), p. 259). When this is done as in text-fig. 3, following the last action-current in the galvanometer string the mechanical curve continues its course for a short time, at the same level, and then begins to relax in a concave curve similar to the relaxation of a motor tetanus (text-figs. 3 and 6). Compared with the relaxation from the plateau of a motor nerve tetanus of the same duration two distinct differences are always observable:—

1. The plateau remains level (plateau after-action) for a much shorter period than the corresponding motor tetanus (at about 35 σ a second), in this instance for 35 σ compared with 90 σ .
2. Once mechanical relaxation begins, the reflex falls more slowly than the motor tetanus at a high rate.

From what has previously been said, the motor tetanus at a high rate of

stimulus must have a plateau after-action only a little less than the after-action of the single twitch, unless in fatigue. Fatigue effects however play no part in this very short plateau after-action of the stretch reflex, because it is the same whether the reflex has only been elicited for a fraction of a second or for a long period. Clearly the preceding reflex discharge could not have been a short series of repetitive impulses at a high frequency, for the plateau after-action would then have had the marked prolongation characteristic of such short motor tetani (*v.s.*, p. 259).

If the later part of the mechanical decline of the totally inhibited stretch reflex be compared with the decline of a short tetanus of high rate (text-fig. 3), it is found that the former (reflex) decline follows the same curve, but that it occurs too early. The relaxation from a twitch or from a tetanus of slow rate begins later but fits with the latter part of the reflex relaxation.

Judged by its relaxation under full and immediate inhibition, then, the stretch reflex appears to differ widely from the purely "motor" reactions. Yet there is one way in which the first may be a derivative of the second. At the cessation of a motor tetanus the excitation of all the muscle fibres lapses at the same instant. In reflex action the individuals composing a motoneurone group do not necessarily discharge strictly synchronously, their individual discharges may hardly synchronise at all. For instance, the early onset of fall from the reflex plateau may mean that a set of muscle fibres, which, though taking part in the contraction, had not received an impulse for some time, was prevented from receiving its next impulse by the inhibitory stimulus. If a group of such fibres had been innervated at a rate of, say, 10 a second and had received their last impulse 90 σ before the onset of inhibition, and its plateau after-action at 10 a second were 120 σ , then it would relax 30 σ after the onset of inhibition. The slowness of the latter part of relaxation could be due to the relaxation of other fibres which had received their last impulse later, and as we have seen this part of the curve fits with that of such a group, if it also was innervated at about 10 a second. The plateau after-action and angle of these latter fibres would be obscured by the relaxation of the fibres which received their last impulse earlier.

In text-fig. 3, the relaxation during a complete inhibition (curve *a*, plotted from fig. 1, Plate 11) is clearly related to the curve of decline from a short tetanus of high rate (*b*, *d*), but its early occurrence shows that such a short tetanus has not immediately preceded the inhibition in curve *a*. The relaxation from the plateau of a tetanus at high rate (*c*) is a different curve. The curve of relaxation from a tetanus of slow rate (under 35 a second) is the same

as the curves *b* and *d*, but is earlier, covering the curve *a* for an equivalent tension except at the rounded commencement of the latter.

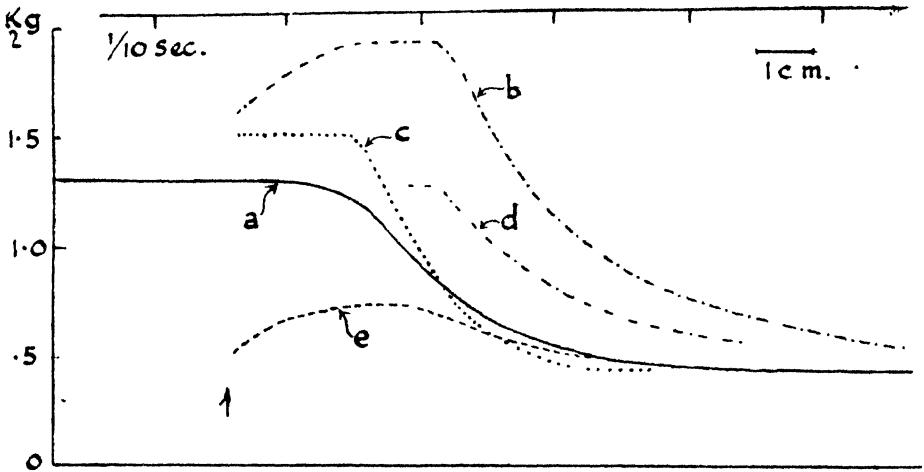


FIG. 3.—The curve of relaxation from a stretch reflex following sudden and complete inhibition with action current cessation at (*a*) plotted with (*b*), the curve of a short motor tetanus at a rate of 50 a second (7 shocks), (*c*) a submaximal tetanus allowed to go on to plateau height at the same rate, (*d*) a smaller contraction produced as was (*b*), and (*e*) a twitch contraction from one break-shock. Each response plotted with the termination of the last action current at the arrow. *M. soleus*. Decerebrate preparation (intercollicular section).

The relaxation and after-action of the stretch reflex would therefore agree with that of a motor tetanus of slow rate, so arranged that no two large sets of fibres received their impulses at the same instant.

Of other explanations for the divergencies from relaxations from a motor tetanus, that which involves the participation of the sympathetic nervous system is negatived by the fact that resection of the lumbar sympathetic ganglionic chain does not alter the curve. Any hypothesis which supposes that the dorsal root exerts some influence upon muscle relaxation, as suggested by Trzeciecki (54) and Ranson (44), derives no support from experiments in stimulating (*a*) the peripheral cut dorsal root during a motor response elicited by ventral root excitation, or (*b*) an inhibitory afferent nerve during a motor tetanus with the dorsal root supply intact and the ventral roots cut or uncut. The motor tetanus is entirely unaltered by either procedure. That explanation which supposes some influence other than the nerve impulse descending the ventral root motor fibres suffers from the defect that no such influence, other than the much too slow process of degeneration, has yet been demonstrated.

The stretch reflex then, in its relaxation, behaves as would a motor discharge of repetitive frequency just sufficient not to permit relaxation of the muscle. The relaxation is constantly of this type, and so there are always units which are just approaching relaxation and require another impulse to keep their tension. Units, if any, which may have a frequency of discharge of over 38 a second must be so few in number that they are obscured at the beginning of relaxation and do not obscure the delayed late relaxation.

There is no evidence from this method of analysis to show whether the units which are due to receive a discharge at any given moment are the same ones which would relax almost immediately, did that discharge not arrive, or whether new units by their arrival make up the defect. Further evidence on this point will be discussed in the next section.

A stretch reflex can be evoked in a quiescent muscle by application of stretch, and inhibited totally in any part of its course. If the analysis be applied to the response during the stretch of the muscle, the type of discharge indicated depends upon the rapidity of the stretch. A slowly applied stretch produces a discharge which gives the same type of after-action from shortly after the onset of stretch to late in the plateau. A rapid stretch (over 1 mm. per 0.04 second) gives a response which, if inhibited very early, leaves an after-action of a summated excitation. For example, in one case the response consisted of three large irregular waves, over a period of 40 σ in all, before complete inhibition silenced the discharge abruptly and completely. The after-action was 180 σ , compared with 150 σ for the twitch, and with 260 σ after 5 shocks at 50 a second. The muscle fibres had therefore probably received two impulses during the 40 σ , since more in that time (*e.g.*, 4 at 100 a second) would have produced an augmentation typical of the 5 shocks at 50 a second. Later phases of a rapid stretch give a slow after-action and relaxation typical of the tetanus at under 35 a second. The rate is here probably nearer the critical speed, for although the relaxation does not alter, the plateau after-action is longer, indicating a nearer approach to the motor tetanus in synchronism. This reasoning therefore leads one to suppose that during the application of stretch the rate of discharge can rise, if the increase of stretch be sufficiently rapid, but not to a rate above 35 a second.

(d) *Crossed Extension*.—Three other types of reflex excitation in this muscle may be compared with that of the stretch reflex. In the reflex excitation of crossed extension, if the reflex discharge be allowed to continue until a plateau is reached before inhibition is allowed to take effect (text-fig. 4) the decline never shows the very early onset characteristic of the decline of the stretch

reflex, but the onset is nevertheless rounded and commences within the period of twitch duration. The delayed late decline and its time of occurrence

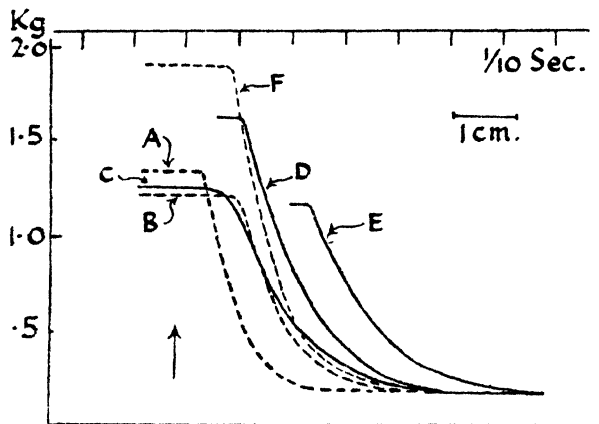


FIG. 4.—Curves of relaxation of crossed extension reflex in *M. soleus*. Decerebrate preparation. Motor relaxations superimposed. A—Relaxation of de-afferented muscle after sudden complete inhibition of crossed excitation. B—Relaxation of the same de-afferented muscle after a crossed excitation lasting several seconds. C—Relaxation of muscle after sudden complete inhibition when afferents were intact. D—Relaxation of the muscle after a motor tetanus produced by a series of break-shocks at a rate of 13 a second applied to the motor nerve. E—Relaxation of a short motor tetanus at a rate of 100 a second. F—Relaxation from the plateau of a motor tetanus at a rate of 50 a second. The curve in each case is plotted with the termination of the last action current at the arrow.

suggest a rate of discharge below 35 a second as in the stretch reflex, although here the exciting afferent for the reflex is being driven by 50 break-shocks a second. Series of tests after varied intervals of discharge revealed at no time the augmentation of after-action caused by the same period of motor tetanus. The after-action of a short reflex regularly presents a sharp angle (fig. 2, Plate 11) which must mean that a majority of the fibres had ceased activity together. The cessation of discharge could have been spread over 15 σ with still a defined angle, and so the curve of a short crossed extension such as that in fig. 2 could be due to two or three evenly spaced waves of excitation reaching the majority of muscle fibres at approximately but not exactly the same time in each fibre. A succession of waves at a rate above 35 a second is not possible owing to the lack of marked augmentation.

The relaxation of the fully developed crossed extension after inhibition differs from the relaxation of the stretch reflex only in the sharper onset of fall in the crossed extension, the slope of fall being the same in the later portion of both reflexes. This is explicable on the assumption that while both are

innervated by a rate of discharge lower than 35 a second, the crossed reflex is the more rapid, *e.g.*, 20-25 a second, the stretch reflex being the slower, *e.g.*, 5-15 a second. The early beginning of fall from the plateau in the latter would be a consequence of the wider spacing of its impulses.

Post-inhibitory tonic rebound and the after-discharge of a crossed extension are both indistinguishable from the stretch reflex, in plateau after-action and in type of relaxation.

(e) *Reflex Discharge in a Muscle Deprived of Afferent Nervous Connections.* - The reflex discharges so far described have been investigated in decerebrate preparations with intact muscle afferents. The de-afferented muscle responds in a different manner. Here the stretch reflex is, of course, not obtainable, but crossed extension forms a ready subject for investigation. As is shown in text-fig. 4, this reflex gives, when inhibited, the after-action typical of a contraction driven at a rate which causes a sustained plateau after-action shorter than the twitch. The late relaxation shows no late delay and follows that of a motor tetanus of high rate. In text-fig. 4 the relaxation of a de-afferented muscle (A) inhibited shortly after reaching the plateau of a crossed extension is compared with that of the muscle with afferents intact (C) under the same conditions. The resemblance of the fall (A) to the curve of relaxation from a motor tetanus at 50 a second (F) is here obvious and the relationship of the fall from the reflex in the muscle with intact afferents (continuous line C) to the relaxation from a motor tetanus at 13 a second (D) and from a short tetanus at 100 a second (E) is also clear. The de-afferented muscle, therefore, by this criterion responds at some rate which is relatively rapid (over 35 a second) and this rapid rate appears to be maintained in the majority of fibres, for when the curve A is compared with the curve E the former is found to be completely relaxed when the latter is beginning relaxation. Furthermore the reflex in the de-afferented muscle, if allowed to continue for more than a few seconds, shows longer plateau after-discharge (text-fig. 4, Curve B) although still relaxing as from a rapid discharge, an effect typical in a motor discharge at high rate which has gone on to fatigue. It is doubtful if it is possible to fatigue soleus by continuation of a stimulus repeated at rates of under 25 a second provided the vascular supply is intact.

The mechanical reflex if inhibited shortly after its commencement shows no augmentation of after-action, but behaves as does the reflex when the afferents are intact. The spinal discharge in the de-afferented extensor, therefore, begins at a rate approximating to, but less than, 35 a second, and later exceeds this rate.

(f) *Discharge in Rapid Muscles*.—The pale, more rapid, muscles when they occur in slower types present slightly increased after-action for an early second stimulus, but the intervals concerned fall within the limit of error caused by slight differences of fibre contraction duration in the one muscle, while the early tetanic augmentation so prominent in red muscle, is not recognisable.

The stretch reflex in these muscles begins to relax after complete inhibition much more early than does the motor tetanus even at 50 a second, and it would therefore appear that in these rapid muscles the discharge is such as just to maintain an even plateau (about 25 a second for an asynchronous discharge), the ripples obtained in motor tetani of regular rapid rates up to 70 a second balancing out, owing to the alternation of units. It is found that their relaxations, as in the red muscles, are unaffected by sympathetic stimulation or excision.

(g) *Conclusion*.—This examination of the mechanical aspect of the stretch reflex therefore reveals that it can be wholly accounted for by assuming the presence of an especial type of motor discharge of slow rate appearing in units out of time with each other. Not only are all the mechanical phenomena of reflex contraction in a muscle of relative purity of twitch duration able to be explained as due to the conventional contraction process of twitch and tetanus, but there is no evidence that the sympathetic nervous system has any effect on the mechanical mechanism. The effect of section of the dorsal root innervation (and this applies also to complete removal of the dorsal root ganglia supplying the muscle) is to modify the process only in such a way as can be explained by an increase in discharge rate of the motor units.

3.—*The Action Currents Accompanying the Stretch Reflex.*

(a) *The Interpretation of Asynchronous Series*.—It is a well-known fact that the reflex of spinal flexion following stimulation of a selected afferent at a high rate can drive the motor units of the flexor muscles so that they respond to their utmost mechanical effect. This type of response, *e.g.*, of the *m. semitendinosus* from the peroneal nerve, when the latter is stimulated by a repetitive stimulus at a rate higher than 50 a second (12), shows, for rates of stimulus, from 60–160 a second according to the type of preparation, an action-current rhythm which is composed of regular waves of a height similar to that of the action-currents of the maximal motor tetanus of the muscle. Above the limiting rate of 60–160 a second the action-current waves become smaller and irregular as Adrian and Olmstead (3) have shown. The size of the action-current in a motor contraction can be used as an index of the number of fibres

in which it occurs (Watts (56), Craib (14)), and in this case the size of the action-currents of the reflex being regularly of size similar to that of the action-currents of the response from motor nerve stimulation at the same rate, the muscle fibres in the reflex can be assumed to be receiving regularly the same rate of efferent discharge. Cooper, Denny-Brown and Sherrington (12) found that different afferents when stimulated with maximal strength at rates above 50 a second could produce different degrees of reflex activity of the muscle, and since it is found that in these circumstances the ratio of the size of the members of the action-current rhythm to the size of the waves of the maximal motor response is approximately the same as the ratio of the size of the accompanying mechanical responses, the muscle fibres receive the same regular discharge which is passing over the afferents evoking it. Such partial contractions were therefore called "fractions" since they must involve only a fraction of the total number of motor units for the muscle, in discharge at rates which certainly follow the rate of the afferent stimulus up to more than 50 a second.

The size of the waves in the action-current rhythm of the crossed extension reflex (unless de-afferented) and in the stretch reflex bears to the size of the action-currents of a whole synchronous motor discharge no ratio which can compare with the ratio of the sizes of the mechanical responses which they accompany. In these reflexes the action-currents are always small and irregular as compared with the size and regularity of their mechanical accompaniment. If the deductions from the mechanical decline of the inhibited reflex described in the previous section are true, then this smallness and irregularity can be explained by the rate of discharge being slow and lacking "togetherness" (synchronism), so that the electrical changes never summate completely at any one instant.

Soleus is typical of all extensor muscles in its asynchronous action-current stream, and the small size of the muscle permits a striking demonstration of single unit responses. If the surface of the muscle be carefully cleaned of perimysium between and around the two galvanometer leads, it is possible, with minimal stimuli, to detect simple regular action-current rhythms. As will be explained later, there is a process of synchronisation of discharge, namely clonus, which makes it generally impossible to judge by the regularity of the rhythm alone whether there is one unit (group of muscle fibres innervated by one neurone) discharging, or many, unless either the size of action-current or the mechanical tension for one unit be known. In practice the mechanical tension developed proved the only reliable criterion of single units, owing to

the varying size and polyphasic shape of the action-current of such single units according to the position of the leads.

In fig. 3, Plate 11, is shown such a single regular action-current rhythm, resulting from the application of a light stretch to the muscle, and its overclouding in the general rhythm when the stretch is increased. This is undoubtedly a single unit discharging rhythmically at a rate of 7 a second. In fig. 4, A, and 4, B, Plate 11, the same unit is seen to be discharging at a similar rate, but slight passive additional length has brought in another unit discharging at from 5 to 5.5 a second, and the resulting waves pass and repass one another.

A weak break-shock to an ipsilateral nerve causes a lapse in the series, with a later reappearance and acceleration (fig. 5), and this is interpreted to be simple weak inhibition with rebound. A stronger stimulus causes complete cessation for longer or shorter period according to its strength.

Such a unit can be caused to discharge by increasing the passive tension very gradually (fig. 7), and caused to cease discharge by releasing the tension. The manner of its onset is found always to be a sudden assumption of discharge rate, however slowly the tension be applied. Such a unit can be followed often for long periods after further application of stretch when many others have joined it. In fig. 6 a gradually increasing stretch involves a series of units, which are seen in the records of two galvanometer strings leading from different parts of the same muscle. Each unit is sudden in onset of discharge and keeps an approximately even rate despite the continuously increasing tension. It is therefore apparent that the growth in size of the reflex response is a "recruitment" (32) of new motor units, each preserving an approximately even rate of discharge. Recruitment of the units is a constant finding, and increase of rate, to the extent found by Adrian and Bronk (2) in the phrenic discharge, does not here occur.

Similarly gradual release of stretch causes a de-recruitment of units, each dropping out suddenly in turn (fig. 8). The only large alterations of rate which have been seen, beyond the slight natural variation of within 10 per cent. in interval, were the missed penultimate beat, which is found rarely to precede a cessation of discharge, and infrequent cases of slowing such as that in fig. 8 (6.6 to 4.6 a second).

During maintenance of the reflex, with a few units discharging, the rhythm militates against Forbes' interesting suggestion of rotation of units (19). The fibres are found to maintain their discharge without dropping out and without new fibres coming in. No sign of rotation of the elements taking part has ever been found to occur and the cessation of the discharge of one unit means the

onset of relaxation. The rate is seen to vary between 5 a second and 20 a second, and discharge at a more rapid rate has never been found.

These minimal fibre responses confirm entirely the findings of the method of evaluation of the mechanical responses. I am informed that Dr. Adrian has also found slow rates in the stretch reflex by recording from leads from the ventral root fibres by means of an amplifying apparatus.

The inhibitory interval, the "silent period." of the tendon jerk (15) can be examined in this way, and is seen in typical activity in fig. 9. It will be noted that here the unit discharging before the occurrence of the jerk lapses during the mechanical jerk, thereafter reappearing at a rate which is more rapid for a long period. The lapse is longer and the after-acceleration more marked, with larger jerks. In fig. 20 is shown the discharge caused by a very light tap to the tendon. The units excited by the tap are so few that the silent period does not exist, the interval between beats being unaltered, while the tension rise is *nil* and after-acceleration does not occur.

A stimulus which causes rebound can be graded to produce rebound in a few units only, and the sudden discharge of several units at different rates, with subsequent dropping out, is seen in fig. 15. The occurrence of the first excitation here, 85 σ after the inhibitory break-shock, is a synchronous excitation of both fibre groups. It will be noted that the larger group undergoes a rapid acceleration and deceleration in its first two beats, thereafter slowing gradually until sudden cessation occurs. The onset of rebound here is almost synchronous in both units, but in various cases various degrees of lagging of some units occurs. This synchronisation by rebound is especially evident when three or four units already discharging are synchronised by rebound, subsequently again becoming asynchronous. The synchronising effect of a short lasting inhibition is of considerable theoretical importance in the genesis of clonus and will be discussed later.

A weak repetitive ipsilateral stimulus can inhibit some groups without affecting others. In fig. 11 groups *a* and *b* are seen to cease discharge after one delayed beat for *b* and two for *a*, leaving one background unit unaffected.

The reflex of crossed extension in response to a repetitive stimulus begins as does the stretch reflex, but recruitment is so rapid when it does occur, that it seems impossible by this method of eliciting minimal responses to find what is occurring during the plateau. This was accomplished by previously sectioning some of the ventral root supply (*c.g.*, L. 6. S. 1, 2 for soleus) leaving the afferents and one ventral root (L. 7) intact. The remaining motor root could be further cut down filament by filament until few enough responding units were secured,

and the action currents again led from the muscle as with the minimal unit method. In this case the discharge in the stretch reflex was still recruiting in type, as is the normal, though it was distinctly evident that some units, themselves discharging slowly, caused a short subsequent inhibition of the others (fig. 12, A), although those others discharged regularly when alone (fig. 12, B). With rapid stretches the discharge became irregular, and in places rapid, presumably owing to the irregular effect of a few of these inhibiting units. A crossed extension after partial ventral root section shows, in response to a stimulus rate of 50 a second, a more or less regular response at 25 a second, even with maximal strength of stimulus (fig. 13), and here again the "inhibiting unit," if present intact, will appear and cause the others to cease for an interval following its discharge. This inhibiting unit is thought to be one which involves a muscle spindle (15), and in these partial motor experiments it shows a tendency to irregular discharge which is never seen when all fibres supplying the muscle are intact.

The muscle with only a portion of efferents intact therefore shows signs of abnormal irregularity and, both in crossed extension and in the onset of stretch, a slightly more rapid type of discharge. The excitatory afferents in such a muscle are capable of causing the stretch response, and their effect can be further demonstrated during an excitation by stimulus to a crossed nerve by causing a sudden stretch or a stretch release (fig. 14). If the stretch be sufficiently sudden the rate of discharge will rise to 50 a second for a short period, if the fall be sufficiently sudden a corresponding lessening of rate occurs.

If the preparation be de-afferented by section of all dorsal roots supplying the muscle, the resulting discharge of crossed extension, if examined for the minimal response with all ventral roots present, shows units in discharge at slow rates of from 16 to 25 a second, with an occasional doubled beat with an interval of some 10 σ . If the ventral roots be cut down, and one unit be followed through the course of contraction, this unit almost invariably begins discharge in response to a crossed stimulus by a double beat of small interval (less than 10 σ), thereafter discharging irregularly and singly at rates which depend upon the strength and rate of afferent stimulus. In the example shown in fig. 16 the discharge, in response to a stimulus at 50 a second, after the double beat rises from 16 a second to 21 a second after 2 seconds, and to 27 a second after 3 seconds. This type of discharge is characterised by irregularity and increase of rate from the onset (figs. 16 and 17), to a maximum, from which a gradual decline in rate occurs if the stimulus lasts too long. Cessation is usually abrupt after slight deceleration (figs. 16, 17, 19). The rate of

discharge bears no constant relation to the rate of exciting stimulus, and though at times following at the full 50 a second, or for a period halving the stimulus rate (fig. 17), it is in general independent, and seems to have an optimum maintained rate at from 35 to 40 a second. A stimulus of 60–100 a second does not secure any discharges with intervals corresponding to these rates, the rate rising to reach its maximum, although the discharge in general is more rapid with a more rapid stimulus. The slow regularity of the motor unit when its afferents are intact is never seen in the de-afferented unit, which tends to slow double beats of short interval (fig. 19) when the stimulus is weakened to secure a minimal effect.

An inhibitory stimulus applied during the course of such a discharge can cause the action currents to cease abruptly, and if the inhibitory stimulus be strong, cessation for a long period after its removal. By suitable adjustment of the strengths of the excitatory stimulus and the inhibitory stimulus the rhythmic discharge of the unit can be caused to drop to a lower rate with the onset of inhibition. Two instances of inhibitory slowing are shown in figs. 15 and 18, and in both of these the rate of exciting stimulus is 50 a second and the rate of inhibitory stimulus 65 a second. In fig. 15 the previously irregular rate of from 29 to 35 a second for the larger unit falls to intervals of 65 σ , 90 σ , and 85 σ , the slow rate lasting for some time after the stimulus, where other units confuse the action-current shape, until recovery occurs to a rate of successively 28, 26 and 23 a second. If the waves of the exciting stimulus have at any moment an optimum period in relation with the waves of the inhibitory stimulus, that period should recur regularly at 66 σ intervals during the period of double stimulation. Two beats during the inhibitory period in fig. 18 do approach in fact such intervals, but also vary considerably beyond them, as do those in fig. 15 (65 σ , 90 σ , and 85 σ into the post-inhibitory period). This independence in rate must be due to a process of summation, and argues, as does the optimum rate, against direct transmission of afferent waves of excitation. The phenomenon is best explained by the assumption of excitatory and inhibitory agents, which are of lasting central duration, such as the E and I of Sherrington (51). The occurrence of these independent discharge rates in excitation and inhibition is in fact strong evidence for the existence of central excitatory states, lasting infinitely longer than the time which one wave can take to traverse the centre.

The short cessation of discharge following a break-shock excitation of a discharging de-afferented motor nerve (15) has been observed in the case of discharge of a few units, and there appears as a silence which may last more

than twice as long as the interval of any two beats in the previous discharge. It therefore appears not to be in the nature of an absolute refractory period for the units, but rather a process of momentary exhaustion of the accumulated exciting agent at the central unit. It occurs alike in flexor and extensor units and lessens in length in proportion as the rate of discharge increases.

These analyses have so far been described for soleus, a slow muscle. In the pale, more rapid, muscles, of which vastus internus is a more favourable example for minimal leads than gastrocnemius, the rate during the stretch reflex appears to be exactly the same as in the corresponding red muscle, and in the de-afferented muscle to reach a similar rate (fig. 21) with the same characteristic acceleration and irregularity. Units have been observed in both types of muscle when de-afferented to reach a rate of 100 a second, or even slightly more, for two successive beats, but the average rate, as has been shown for the red type, is very much lower; it remains for further investigation to reveal whether it is possible to drive the units at more rapid rate. It is apparent that in the reflex used as test, namely, crossed extension, a large amount of concurrent inhibition is produced by the same stimulus which excites that reflex, and this may account for the slow average rate in this reflex.

(b) *The Interpretation of Synchronous Series.*—There occurs in the fully-developed stretch reflex a phenomenon, called "clonus," which consists of a regular discharge most commonly set in action by a tendon jerk (55, 15). In a preparation showing good rigidity it is a common occurrence, and in these often occurs spontaneously. In the latter case the smooth mechanical plateau of the reflex gives way to a tremor, and this in turn to a smooth mechanical plateau again, such alternate clonus and fused tonus alternating at fairly regular intervals. A record of such a transition reveals that the myograph curve during clonus becomes a series of partially fused twitches, while the action-currents, hitherto irregular and small, become regular and large. Similarly a stretch reflex elicited in such a preparation shows the transition (fig. 22). This change of action-currents is reversed in the reversed transition and is in all respects the same as the change from the clonic after-discharge of the tendon jerk to tonic discharge (fig. 23), which, it has already been argued (15), is a change from a synchronous discharge to an asynchronous one. This is further supported by the fact that the relaxation of each from a given tension is the same, except for the early occurrence of the angle in the latter. The action-currents of the fully-developed clonic type are large and simple and truly proportionate to the size of the mechanical response. The silent intervals

between them are considered to be equivalent to the silent period of the jerk and therefore all the discharge is represented by the large waves, and from their size and regularity it is considered that the rate of discharge in any fibre is that of the rate of clonus. Clonus is in fact simply tonic discharge thrown into "togetherness" (synchronism). It is a common feature that a tonic discharge will continue for long periods in a state of partial synchronism (fig. 23) where the discharge of all the units in activity is grouped, though not synchronous, and indeed all shades of variation from complete asynchronism to perfect synchronism can be found, representing all the stages in the discharge which is alternating from tonus to clonus.

Two conditions predispose to clonus when clonus tends to become asynchronous, namely, isotonic recording (allowance of greater shortening), for the more resistant the spring the more difficult is clonus to evoke, and in the second place the degree of development of the stretch reflex. The first of these has the effect of shortening the duration of the plateau after-action of twitch and tetanus (23a) with consequently more relaxation and more subsequent rise between the plateau of one beat and the next. Greater shortening therefore involves greater range of tension change and greater movement of muscle elements for each beat. The second condition, the degree of development of the stretch reflex, can be shown to be dependent upon a certain optimum tension. This tension must be active and does not depend upon length, for the tension may be brought to the same level at a shorter length by means of a stimulus to a crossed nerve, and clonus begins at the same tension.

Given an optimum degree of stretch reflex, the first factors, range of tension change and degree of movement of muscle elements with each beat, can be examined separately. It has been shown (15) that in the clonic after-discharge of the tendon jerk each efferent wave can be set up before relaxation begins. This is evident also in spontaneous clonic discharge as in figs. 22 and 24, when many beats occur before, or at the angle of, the beginning relaxation of the previous plateau, and must therefore be set up by some afferent discharge which reaches the centre several σ before relaxation from that plateau. This is especially evident with the most strict isometric conditions. The cause of this reflex excitation must therefore lie in the plateau of the beat or in its previous onset and rise. Adrian and Zottermann (4) showed that the stretch end-organ in the sternocutaneous muscle of the frog responded most rapidly during the onset of stretch and tended to adapt but slowly during the plateau. The acceleration of discharge was most rapid for the stretch of most rapidity and amplitude. There is ample evidence for the existence of such an organ in

mammalian muscle (47, 48, 50) and in the condition of clonus its greatest acceleration of tension change should be spread over the period from the moment it is arrested from the fall in tension of the previous beat to the end of the period of rapid rise in tension of the following beat. In the plateaux the rate of discharge should be more rapid (*i.e.*, less adapted) if interrupted by intervals of relaxation and reassertion of tension. The effect of greater tension range on the tension end-organ must therefore be a greater intensity of afferent excitation at the onset of each beat.

Hoffmann (28) described a cessation of discharge following a tendon jerk and considered it to be an inhibition. In another paper (15) I have shown that this "silent period" is due to an inhibitory proprioceptive volley produced by the wave of excitation of the muscle fibre. The silent period between the beats of clonus is regarded as the expression of a central inhibition, produced in the same manner as the silent period of the tendon jerk, and the difficulty of eliciting a tendon jerk during the early part of a clonic silent period is evidence that the two are identical. Each beat of clonus must therefore be a recovery from a period of inhibition, and the fact that shortening *per se* can influence the tendon-organ excitation mainly at the beginning of the beat, and yet predispose to clonus, therefore reveals that the ensuing beat excitation has in part been derived from the onset of the previous beat. It may indeed have been derived in part from the relaxation of the penultimate beat, but in the absence of any demonstration that *relaxation* of tension, active or passive, affects the discharge of any end-organ except by its effect in causing deceleration of the tension receptor (Adrian and Zottermann (4)), it is preferable not to regard relaxation of active tension as an exciting stimulus. The occurrence of some beats over 6σ after the angle of the previous response means that the tension afferent excitation arriving at the central units has lessened because of the commencing relaxation just before causing discharge of the units. This is but evidence that the summation of excitation, from the previous ascent and plateau, is sufficient to cause rebound when the inhibition diminishes in spite of the absence of late accessions of excitation. This long lasting central effect of tension excitation is seen in the long period of acceleration of one unit after a tendon jerk (fig. 9), so that it is possible that the greater accumulation of central excitation occurring with greater shortening is without effect until the silent period wears off, when it results in greater, more synchronous rebound.

In clonus, therefore, the type of discharge can be described as a series of synchronous rebounds. Since the synchronism must depend upon the degree and synchronism both of the preceding proprioceptive excitation and of the

preceding proprioceptive inhibition, the explanation of the facilitation of clonus by a tendon jerk is obvious from the fact that it provides both these phenomena in purest form.

On this view clonus cannot take place unless the recovery from any silent period occurs at approximately the same moment in all the units which are taking part. This is the reason why the tendon jerk with a marked "hump" indicates a tendency to clonus. A muscle may show spontaneous lapses in synchronism in one beat (fig. 23), or irregular changes in successive beats (fig. 25), or long wave-like variations in synchronism, which constitute alternating spontaneous tonus and clonus. The processes concerned are evidently multiple, for it is evident that the process of balanced synchronised rebound depends upon a nice equilibrium of rise and decline of both excitatory and inhibitory states at all units in the centre. A certain degree of tendon-organ excitation is necessary, and a certain proportion of this must be active, so as to provide a degree of inhibition. It is therefore apparent that the optimum tension for clonus should necessarily be partly active and partly passive tension.

Two conditions each predispose more than any others to present clonus at almost all tensions. Of these the first is the clonus of the spasm produced by tetanus toxin, and this question must be left for treatment elsewhere. The second is the "spinal" state occurring in the lower segment of the transected spinal cord after the subsidence of the condition of spinal shock. In this latter case the rhythm is perfect at low tensions although the synchronism of the beats is irregular (figs. 26 and 27), while at high tensions there is a tendency for some units to break through the silent period, leaving the general rhythm intact. The mechanism of clonus is undoubtedly spinal, as the records of Viets (55) show, and fig. 27 reveals that the spinal stretch reflex described by Denny-Brown and Liddell (16) is essentially clonic in nature. The sympathetic innervation of the limb is not in any way essential to the process.

4.—The Distribution of the Postural Reflex.

(a) *The Extensor Response.*—It was early shown (49) that the rigidity of the decerebrate animal involves mainly the extensors of the limbs. The extensors, however, are themselves multiple muscles, being composed of heads of different fibre length and colour. The question of the red muscles, being too long and involved to consider here, is being dealt with in another paper, but it may be taken for the purpose of the present discussion that each extensor group of ankle, knee, hip, elbow, and shoulder, and probably also all other joints, in all animals, possesses a "red," slow muscle fibre group. Thus in the ankle-extensors

soleus is the red, slow "head," and gastrocnemius the rapid "head," and of the knee-extensors crureus is the red, slow head, while the rapid heads range in duration through vastus internus and rectus femoris to vastus externus. Now it is especially important in view of the hypothesis put forward by Hunter and Royle (29) that the relation which the red, slowly contracting heads bear to the effectors of the postural reflex be examined.

The approach to this question is best by examination of the stretch reflex in two typical heads, such as gastrocnemius and soleus. Stretching these muscles before and after cutting the motor nerve in a decerebrate preparation reveals that both muscles respond to passive stretch with a reflex contraction. The presence of this contraction is simply revealed by a strong inhibitory stimulus or by section of the motor nerve, and its presence in each is accompanied by an irregular stream of small action currents (15).

In a previous paper (15) it was explained how the tendon jerk in a decerebrate preparation was an index of the number of motor units available for the stretch reflex, and how as the reflex was intensified by additional stretch more and more motor units were brought into activity, with the result that while the action-current of the jerk remained for a certain range approximately the same size while the mechanical jerk grew less and less. This was interpreted as evidence of the recruitment of more and more units by the increasing reflex, and it has now been described in the last section how increasing the stretch reflex merely increases the number of units involved, while their rate of activation, being slow, the tendon jerk efferent volley is but little altered. By measuring the tension fall produced by maximal inhibition it is found that in a muscle such as soleus further stretch does not evoke appreciably more reflex tension beyond the level where the maximal tendon jerk is occluded mechanically. The maximal tendon jerk, though involving only a fraction of the motor units, seems therefore to involve just those which are capable of excitation by the greatest sustained stretch of the muscle. The size of the maximal tendon jerk (mechanical and electrical) is therefore an index of the fraction of the muscle open to a stretch reflex under these conditions in a decerebrate animal. Its size of mechanical response at any tension compared with the response at low tension is an index of the number of motor units available for the stretch reflex which are at the time not involved in that reflex.

Gastrocnemius (pale, rapid) and soleus (red, slow) can thus be tested for the presence of the stretch reflex by three ways, namely, comparison with the same length after cutting the motor nerve, the depth of maximal inhibition, and the

size and occlusion of the tendon jerk. By the first two of these methods it is found constantly that gastrocnemius, in proportion to the maximum tension development of its units, develops a stretch reflex which is very small indeed compared with proportional stretch reflex of soleus. The largest stretch reflex seen in gastrocnemius was of 1500 gm. of active tension, compared with its potential motor tension value of well over 10 kilos., and is small in size compared with even a single motor twitch of the muscle. Soleus, on the other hand, quite often develops an active stretch reflex tension which is in the region of the maximum tension it can develop at a rate of discharge of 15 a second (over 90 per cent. of its maximal tension of 100 a second).

Soleus responds, in decerebrate rigidity, with a stretch reflex response over ranges in length at which it is loose and folded if the motor nerve be sectioned, while gastrocnemius, whether entire or the internal head alone, responds with reflex contraction only when section of the nerve reveals that some passive tension is being exerted on the inactive fibre at that length.

In any given circumstances therefore the red soleus reveals more readily a stretch reflex, and further increase of length can involve more of the muscle in that reflex than is the case in gastrocnemius. This is particularly noticeable when the rigidity is slight, from a posterior section, or early after spinal section, when soleus will show some sustained stretch reflex and gastrocnemius none at all. This relationship also holds between crureus (red) and vastus internus, and the two short heads of triceps brachii.

In the above examination the length is varied and external conditions kept constant. Further data are revealed if the length is kept constant and other conditions varied. If the labyrinth be varied from the maximum to minimum position of Magnus (36) both muscles vary in tension. This variation (text-fig. 5) is present in two forms, the one a rapid twitch-like contraction of all extensors when the head is moved in any direction (? kinetic righting reflex), the other a maintained rise of tension of slow origin, which is the myographic demonstration of the tonic neck and labyrinthine reflexes. The first type involves both red and pale muscles, and in an active preparation with pre-collicular section is seen as much in the pale muscles as in the red, and often involves the former more than the latter. The tonic neck and labyrinthine reflexes are seen to much greater advantage in the short red muscles (text-fig. 5), and, when the reflex response is small, it is seen only in these muscles (text-fig. 5, A, c). When the total stretch reflex is tested in conjunction with variations of the position of the head the maximal stretch reflex is found to alter in amount, being greater in the maximal labyrinthine and neck posture.

The variations in reflex tension when the muscles are stretched to less degree, as in text-fig. 5, are therefore but proportional to the degree of potential

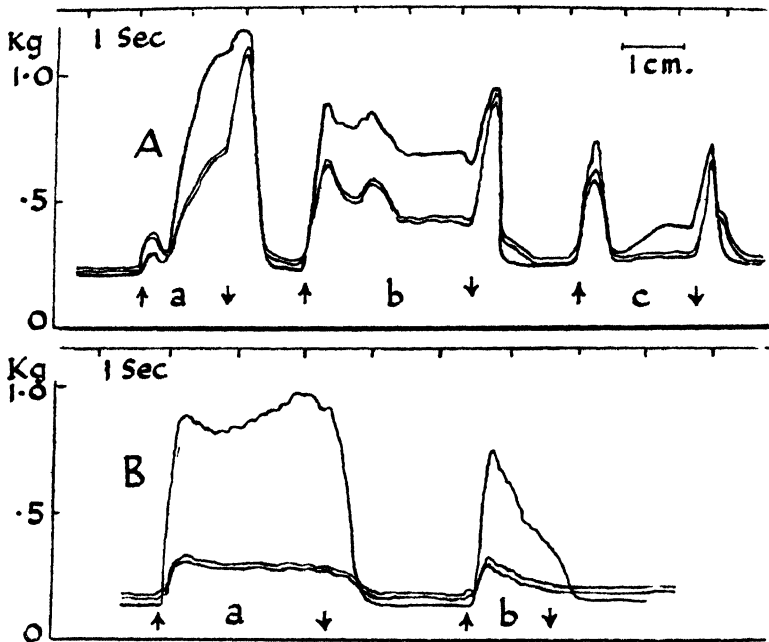


FIG. 5.—The reflex effect of the labyrinthine and neck reflexes on red and pale muscle. Preparation with section just precollicular in level. Double wired isometric myograph with pulls in same direction. A—M. vastus internus (pale) double record (obtained by means of hair on myograph point) and M. crureus (red) single record. Labyrinth maximal at ↑ and head and fore-limbs moved so that labyrinth minimal in effect at ↓. B—M. triceps, short lateral head (pale) double record, short medial head (red) single record. Neck dorsiflexed at ↑ and ventriflexed at ↓. Labyrinth in intermediate position in each neck posture.

stretch reflex present with intermediate head posture. The labyrinthine and neck reflexes therefore act exactly as does increase of stretch, and the two factors mutually facilitate. Again, if the level of brain section be varied both muscles keep their relative degrees of stretch reflex, and vary together according to the degree of rigidity present. In the fresh spinal preparations any sustained stretch reflex which is present is seen in soleus when not in gastrocnemius.

These variations run parallel in all the red and pale muscle pairs, the red crureus and the pale vasti, and the short red medial head and the short pale lateral head of triceps brachii. In the former case vastus medialis is found to occupy an intermediate position between crureus and vastus externus. The red muscles therefore can be said to contain the focus of the stretch reflex (the

bulk of the effectors of low threshold), while the pale muscles contain stretch reflex effectors of medium and high threshold. This is true for increase of the reflex by stretch of a compound muscle and also for external facilitation.

The tendon jerk development is an index of the units available to stretch afferents in the same circumstances. The acute spinal animal, and a decerebrate preparation with lack of posture show good tendon jerks in the red muscles but very little in the pale muscles, a circumstance which indicates a great shrinkage of the potential stretch reflex fraction in these conditions. That no stretch reflex here exists cannot be due to lack of available units, for the tendon jerk, a stretch phenomenon, still can excite some of them, though less it is true. Sustained stretch excites very few indeed. The synchronism of the tendon jerk afferent volley has a powerful central excitatory effect, while the lagged irregularity of the increasing impulses of several tension afferent organs discharging independently is presumably not effective. The asynchronous series of impulses from a sustained stretch in the decerebrate state must, when the passive stretch is powerful, approach the equivalent central effect of a synchronous afferent volley, because a maintained stretch can occlude the tendon jerk in the resulting stretch reflex. The "spinal" state must therefore be due either to some lack of response from the tendon organ (possibly owing to some greater strain on the endomysium and less on the tendon organ *via* muscle fibre, as evidenced by the greater tap required to elicit the spinal jerk) or more probably in greater part a lack of some degree of central excitability, resulting in a greater central distinction between a synchronous and an asynchronous afferent stream.

It happens that in a rigid decerebrate preparation where the stretch reflex is continuously clonic, slight stretch of a muscle produces a clonus of a certain rate, *e.g.*, in a particular instance 14 a second (vastus internus), and a further stretch an increased reflex tension of the clonus still at the same rate, until a level is reached where further increase in length suddenly ceases to change the active tension more than a minimal amount, but increases the rate of clonus instead (up to 20 a second in this instance) and eventually causes a confused rapid rhythm. Here the stretch reflex, given a certain fraction of the motor units by the hind-brain centres (intercollicular section), could recruit certain of those units from zero to maximum by increase of stretch, maintaining in each, as was shown in the last section, a certain type and rate of discharge. Once all units were involved further increase in stimulus evokes but an increase in rate, and with it a tendency to asynchronism (lack of tension-organ acceleration with each beat, owing to already nearly maximal stimulation).

This occurs in mixed muscles as in simple ones, but in the former is complicated by the incidence of tension not falling evenly on the different heads. For instance, in a whole quadriceps it is not uncommon to find that a stretch reflex having been elicited, and the stretch increased so as to involve the whole of the units in this stretch-fraction as evidenced by the occlusion of the tendon jerk and an increase in rate of clonus, a further stretch occasionally makes it possible for another tendon jerk to be elicited, and then a great deal of further stretch is necessary to occlude this jerk mechanically. This is never found in the simple heads, and its explanation rests on the uneven incidence of stretch upon muscle heads of different lengths and different levels of insertion into the patella, for each head has its own stretch reflex.

A similar difference in stretch incidence in a complex muscle, such as quadriceps, must account for some of the sharpness in relaxation after inhibition of crossed extension found by Liddell and Sherrington (33) compared with that of a motor tetanus. The muscles used by these authors (crureus and vastus medialis) gave a relaxation curve from a motor tetanus at 50 a second which is typical of a mixed red and pale muscle, and yet the relaxation from an inhibited crossed extension behaved as though only purely pale muscle had responded. Experiments reveal that the red muscles are of lower threshold for, and respond more completely to, a crossed stimulus than pale muscles, and crureus and vastus internus are typical in this respect. It is found that although the tonic combined muscle will relax under inhibition often much more rapidly than after a motor tetanus, as Liddell and Sherrington describe, the pale head (vastus internus) alone, and the red head (crureus) alone, will, when thus inhibited completely, each invariably relax more slowly than its motor tetanus at 50 a second. The phenomenon described by Liddell and Sherrington therefore is in some way dependent upon the combination of the two heads in their rigid state, and is accounted for by the great range of tonic length in crureus (which behaves exactly as does soleus), and since in this case the motor response was taken from equal initial tension (and therefore increased length) compared with the reflex response, the inhibitory relaxation of the latter does not show the relaxation of crureus except for a slight delay in its later part.

A stretch reflex or tendon jerk of a pale head causes generally a response in the corresponding red head, but not *vice versa*, for a stretch or jerk in soleus or crureus causes, under conditions where direct transmission can be avoided, no effect on the associated pale heads. The relative degrees of reflex response from each head does not, however, depend upon interaction between them,

because each preserves these relative degrees when completely isolated by nerve section.

The difference in duration of tendon jerks in quadriceps in the decerebrate and spinal preparation found by Ballif, Fulton and Liddell (6) follows as being an expression of the shrinkage of the stretch fraction to entirely red, slow muscle in the latter case, for it was explained earlier that the spinal jerk, in a relatively homogeneous muscle such as soleus, maintains, as does the decerebrate jerk, approximately the same duration as a twitch in the same circumstances of length, shortening, and temperature. Similarly the duration of a jerk in the mixed muscle quadriceps is altered by the labyrinth, and in the same way by the neck posture, from a long low jerk in the minimal posture to a high short jerk in the maximal posture, an expression of the extension of the fraction to pale heads.

The stretch reflex in each extensor muscle has some influence over each other extensor muscle. This influence is both excitatory (Shoen (53), Blake-Pritchard (7), Magnus (37)) and inhibitory (15) for the ipsilateral extensors, and excitatory for the crossed extensor (Philippson (42), Sherrington (48)). In my experience of the interaction of the ipsilateral extensors the inhibitory effect is the most common, but the exciting effect can often be seen in the interaction of two clonic muscles. For example, in a clonic decerebrate preparation the clonic quadriceps can be made to beat more rapidly than does a particular tension of clonic stretch reflex in soleus. Yet if both are beating together, and soleus but weakly, a strong tension on quadriceps causes soleus to beat at the same rate, yet making occasional attempts (as at *a* and *b*, fig. 28) to break down to its own slower rhythm. This must be in part due to the inhibition in quadriceps being transmitted to soleus, and this can readily be demonstrated by transmission of the silent period of the former to the latter, but it is in part due to excitation, because such a clonus in quadriceps can occasionally cause soleus to beat when otherwise it is without excitation.

So also this relationship exists between individual heads of an extensor muscle, such as quadriceps, or the combined ankle extensor, though in such cases all excitatory afferents, as judged by the ease of transmission of excitation, seem to converge mainly upon the red, slow head.

(*b*) *The Flexor Response.*—The flexor muscles show no strongly maintained stretch reflex under most conditions, except that in preparations with pre-collicular section they have been found to enter into sustained contractions (Magnus (36)) which have been seen myographically by Dr. Liddell and myself in brachialis anticus as asynchronous action-current rhythms accompany-

ing a smooth maintained plateau. Their proprioceptive origin we have not yet established. Tibialis anticus, an undoubted flexor, frequently exhibits tendon jerks (5) requiring a heavy tap or pluck to elicit them. They conform with the type of the extensor jerk, possessing both silent period and efferent volley of short latency (15) (figs. 30 and 31, *b*). It has also been found that an afferent stimulus which, when strong, is capable of exciting a large fraction of the flexor, when reduced in strength until it is minimal in reflex exciting effect now markedly facilitates the tendon jerk in that flexor muscle (fig. 30). This is but another aspect of the large efferent volley of a tendon excitation obtained during a reflex background (15).

The stretch afferents of such a flexor can affect a large fraction of the motor units of the muscle, if aided by an overlapping extraneous excitation. Alone they can do but little, but they can and do produce a sustained discharge at a few units. With marked decerebrate extensor rigidity and a sensitive galvanometer string with careful leads one has been able to record a continuous clonic discharge, produced by stretch and abolished by its absence (fig. 31, *b*), which can often be appreciated by the hand as a faint tremor in the tendon. This tremor is often felt when the extensors are clonic and exhibiting an enhanced stretch reflex, and so it must be maintained that decerebrate rigidity is not entirely a standing rigidity, but a general enhancement of the stretch reflexes in both flexor and extensor so that the extensor antigravity standing reflex and the flexor stretch reflex are both enhanced. It is also found in the spinal flexor after the subsidence of spinal shock (fig. 31, *a*) when the extensors are clonic. This flexor stretch reflex shows a discharge very much slower in rate compared with the rate of discharge in the same units when excited by a repetitive stimulus to an afferent nerve of the same side. This, however, is an effect which is explicable on the omnipotent central exciting effect of a stream of afferent impulses in synchronism, compared with a series of impulses arriving out of time with one another, as must occur in most natural stimuli.

5.—*The Postural Mechanism.*

The extent of the development of the stretch reflex varies directly with the extent of reflex standing. Both are minimal in the fresh spinal preparation, both are present in the chronic spinal preparation (16). Precollicular section of the brain stem or basal ganglia leaves them both minimal, or slightly enhanced, according to the level of the section, and intercollicular section in the mid-brain accentuates both (Liddell and Sherrington (31)). The labyrinthine and neck reflexes have been shown to influence the stretch reflex just

as they influence the posture of the limb. The stretch reflex is the basis of postural adaptation.

Section of the afferents of the limb abolishes the stretch reflex (31), but it is an early observation that it leaves intact the reflex of crossed extension (50), such reflexes as the scratch reflex (50), and movement elicited by stimulation of the cerebral cortex (39). It abolishes standing, both reflex and voluntary, but leaves a slight degree of reflex stepping.

Ranson (44, 45) has objected to the statement that posture depends upon the muscle afferents on the grounds that extension, of a certain degree of maintenance, occurs after de-afferenting the limb aseptically and allowing recovery, and that the spasm produced by tetanus toxin can occur after de-afferenting. Liljestrand and Magnus (34) observed labyrinthine effects also after de-afferenting. These types of extension do not enable the animal to stand, nor to sit normally; their purpose is not evident in any posture. Propped up on such a leg the animal soon collapses. Ataxia does not explain their lack of purpose.

The labyrinth alone cannot cause any units to maintain a discharge without a modicum of excitatory assistance. This assistance appears to be derived from the stretch organs connected with those units *plus* a modicum of assistance from the excitation derived from release from supracollicular structures. For instance, in the de-afferented muscle the maximal labyrinthine position causes discharge only when decerebrate rigidity is especially well marked in other extensors not de-afferented, and then takes the form of a maintained discharge with irregular rhythm occurring in the focus of the stretch reflex units (fig. 34) and, in the single unit, seen as a few odd waves of irregular frequency. Strong extensor rigidity in the rest of the limb (even well-marked clonus) seems likewise unable to cause the extensor unit to discharge in the absence of proprioceptive afferents, unless aided by a strong maximal labyrinthine influence.

In the extensors the extent of the units excited by proprioceptive stretch afferents is so great that their focus at any particular joint needs but little additional excitation to cause it to enter into maintained discharge without any applied stretch, the small subsidiary discharge itself providing the initial tension to continue the reflex. Such an initial discharge we have seen provided by the labyrinth or neck reflex *plus* decerebrate rigidity in the de-afferented focal unit. So it is that in decerebrate rigidity, even with the limbs free in the air, they are extended. Judged by the action-currents in them their contraction is then very small, only enough to extend the joint, but this amount

is produced by the facilitation of a few of the focal units, and any applied flexion instantly enhances the extension.

The brain stem, the labyrinth and the neck reflexes all can facilitate the stretch reflex by a means which is considered not to differ from any other series of asynchronous impulses in any other reflex path. This facilitation can, at the units it affects most strongly, cause discharge if it be itself strong, even in the absence of proprioceptive excitatory afferents. Such a discharge may be maintained, provided that the head and neck maintain their position, but that discharge is nevertheless not posture, and therefore not muscle tone, as we understand it.

Excitation of a muscle has been shown to cause a proprioceptive centripetal discharge from a receptive end-organ in muscle. The extreme rapidity of the onset of the central inhibition resulting from an efferent discharge (15) indicates that the adequate stimulus to the end-organ is either the electrical action-current of muscle fibre or the change which underlies the action-current. The muscle-spindle is therefore indicated as the end-organ responsible for this type of proprioceptive inhibition. Tension has long been known to evoke an afferent discharge, and this is believed to occur from the tendon organs of Golgi, which seem well adapted to appreciate tension, passive or active. The muscle-spindle exerts an inhibition, the tendon-organ an excitation, upon the centre. These two processes have been shown at play in the process called "clonus," there occurring in each wave as a series of excitations, summing and eventually breaking through a single deep inhibition, which is during this time passing off, the resulting efferent wave causing yet another deep inhibition.

It has been shown that the stretch reflex exhibits two forms, this clonic form and an asynchronous tonic form. The transition between the two, when it occurs, is smooth and uneventful, and the type of discharge in the tonic form is found by mechanical and electrical analysis to be the same as we conceive to take place in the clonic form. But clonus is (according to our ideas) exactly the same as the motor tetanus, in that each discharge of each fibre occurs synchronously with a discharge in each other fibre, and the relaxation curve confirms this.

If this be so then the same afferent events must be occurring in the two forms. The tendon-organ excitation is the same in both except for its regular acceleration in clonus. The muscle-spindle must still sample as many efferent waves in one form as the other, but in the clonic form all muscle-spindles discharge at the same instant, while in the tonic form they discharge irregularly, asynchron-

ously ; but the same amount of discharge must occur in each during a period of say, 0.5 second. Since the stretch reflex needs a stretch to elicit it, a certain steady stream of stretch afferent excitation is, for it, a necessary establishment. Any discharge this will cause, will cause two additional afferent streams, one inhibitory and one excitatory. These three series must then summate, so that the central units accumulate excitatory substance until a discharge occurs, and failing a discharge the conditions revert to the original passive stretch excitation, which *ex hypothesi* can cause discharge. If discharge is too rapid both stretch excitation and repercussed inhibition accumulate in greater quantities. For example, supposing the tendon-organ discharge to increase until the discharge from several tendon-organs firing separately and asynchronously has produced many chance synchronous overlaps at a unit upon which they converge (this must occur the more often, the more rapidly they discharge), then the resulting efferent discharges being more rapid, the muscle-spindles sampling these discharges will return a more rapid inhibitory stream, which will have more chance overlaps at the unit of convergence the more rapid each stream becomes.

When once the stretch-afferent volleys in their series overlap completely in a unit of time the equivalent of a tendon tap is produced, namely, a synchronous afferent exciting volley. And when once proprioceptive inhibitions become synchronous the result is a silent period. A silent period has been found definitely to inhibit a tendon jerk volley until even in its late phase (15), and the refractory period of the tendon jerk is due to this overlapped inhibition. The proprioceptive inhibition can always therefore limit the pace, and it is omnipotent when pitted against the proprioceptive excitation.

Central summation should ensure some inhibitory effect, even from one spindle. In any condition of stretch reflex equilibrium it should therefore be possible to demonstrate the presence of inhibition acting upon the motor units in entirely asynchronous reflexes, and this has been accomplished by removing the stretch, *i.e.*, shortening the length of the muscle, rapidly during a stretch plateau, as in fig. 32. This procedure, though removing the tendon excitation, should yet leave the unit in after-discharge for a brief time, as was seen to occur with the removal of stimulus in a de-afferented unit (figs. 17 and 19). The stretch reflex discharge, however, ceases instantaneously if the fall be rapid. The rigid plastic state of the muscle ensures that the tension on the tendon-organ drops immediately, and accordingly its discharge must lessen immediately. The immediate cessation of central discharge indicates that the excitation had been just exceeding the central value of the proprioceptive

inhibition, for after the onset of release the latter, being altered last, takes immediate effect, and no after-discharge occurs. If the release be slower, so as to ease tension on the tendon-organ gradually, simple derecruitment results. Apparently the excitation thus damped by sudden release is still present in subliminal form and can outlast the inhibition, for it can often sum with the earliest adaptation of the tendon-organ at the new level to produce a rebound of contraction at that level—the shortening reaction. Similarly, during the onset of the stretch reflex the predominance of excitation evokes an intense discharge of higher rate than usual and the resulting inhibition causes a sudden silence and waver on the ascent, or else a fall in tension when the stretch is completed, the lengthening reaction.

The proprioceptive inhibition then is summing at the central units all through the response and opposing there the afferent excitation arriving from the tendon-organs, also summing at each unit. It is essential to the mechanism that each of the inhibitory afferent proprioceptors from any given muscle play upon exactly the same motor units which are covered by each other inhibitory afferent proprioceptor, and further that the effect of each at any one unit be the same. Each proprioceptive inhibitory afferent must completely occlude each other proprioceptive inhibitory afferent from the same muscle. This becomes apparent when it is considered that no unit appears to drop out of contraction when fresh units and fresh inhibition are brought in, and that the rate of discharge is maintained evenly slow in all units, thus indicating an even increase of inhibitory effect at all units through all increase of excitation. This evenness is such that the first units to discharge in a given stretch reflex, the units of low central threshold, reflect a proportionate inhibition, so that when the electrical responses of a minimal reflex are recorded the discharge is evenly maintained. It is only when the ventral roots, and with them some inhibitory sample units, are cut down that it is possible to obtain such a unit which is much above stretch threshold of any other discharging units (the “inhibiting” unit in figs. 12, A, and 13).

The proprioceptive excitation obviously is arranged so as to play more upon some units than upon others, it can excite some units only when aided by the influence of decerebrate rigidity or by the labyrinth, and in the conditions of minimal stretch reflex (spinal shock) it can cause some units to discharge only when all its members produce a volley within a minimal period (tendon jerk) and fails to cause any of these units to discharge when it is asynchronous. This central variation can be explained in terms of summation of the excitatory agent (E).

Thus, taking the tendon jerk as the result of one synchronous afferent volley, the aggregate of units making up the centre of a mixed extensor, assumed to be all affected by the afferent proprioceptive excitation in some way, can be divided into a class A, which includes all those which can be caused to discharge an efferent volley in response to the afferent tendon jerk volley and a class B composed of those which do not. The difference between the jerk with labyrinth maximal, and the jerk with labyrinth minimal, for instance, is then that the numbers in class A are greater in proportion to class B in the former case (when it contains all red units and many pale units), than in the latter (where it contains a few red units). Since in the maximally facilitated jerk the size of the jerk can be varied by the heaviness and direction of the tap to the tendon, and it is still possible by a very light tap to the tendon to excite only red units, it can therefore be maintained that the central effect of the combined tendon excitation is a graded one, being greatest at the focal red muscle units and ranging to its least effect on the pale muscle units. The labyrinthine effect can now be explained by supposing that passing from minimal to maximal it affects each unit equally with increasing degrees of excitatory substance which, summing with any tendon excitation at units previously subliminally centrally stimulated, is sufficient to throw increasing numbers of those units (class B) into class A. Class B in fact forms a large "subliminal fringe" (15a) of units affected by stretch.

So also the influence of the neck reflexes, and the absence of the brain anterior to a precollicular section, is explained by the result of a widespread, evenly disposed, addition of varying degrees of excitatory agent.

It is conceivable therefore that the condition of spinal shock with its suppression of the stretch reflex, leaving a red tendon jerk intact, is a sign of still greater suppression of subsidiary E agent than is the case with minimal labyrinthine and neck reflexes. In this case then the grades of units with highest stretch afferent exciting effect are now not provoked to discharge by an asynchronous afferent stretch stimulus though still excited by a synchronous one.

Similarly the flexor units affected by proprioceptive stretch afferents are graded so that there are few which respond to a synchronous exciting volley from the tendon-organs, and very few indeed which respond to an asynchronous volley, though it is undoubted that with facilitation by subsidiary exciting agent (E) from any source their stretch mechanism can come into general operation.

The pale rapid extensor units of highest threshold for the stretch reflex are

devoted to the kinetic reflexes and these effects will be dealt with in another place. It appears that the tendon excitation and proprioceptive inhibition can reach all of these units although not alone able to excite them even with the maximal rigidity and the maximal labyrinthine and neck reflex effect, for the reflex of ipsilateral extension can alone facilitate a tendon jerk in them.

The necessity that for any given level of maintained contraction a certain proportion of the tension development should be passive (involving all tension afferents) is reflected in the long silent period of a tendon jerk compared with the interval between waves of maintained discharge at the same length. This is particularly evident when a muscle is continually clonic, where the slow clonic after-discharge of the tendon jerk when unoccluded (fig. 23), compared with the rapid background clonus, and the falling away of the tension of the after clonus demonstrate that the degree of active tension reached by the crest of the jerk contraction involves the tendon-organs only partially and their central exciting effect is then not sufficient to balance completely the greater inhibition produced by activation of so many units. The resulting silent period is too long for the duration of this degree of excitatory summation at some units and these de-recruit group by group, and any interference produced by lagging, by lessening the effective synchronism of the inhibition, delays this de-recruitment (as in the asynchronous beat in fig. 23). When the mechanical jerk is nearing occlusion (fig. 25) the passive tension excitation remains the same, and the active tendon excitation undergoes only the periodic acceleration which is the result of the fusion of the clonus still not quite perfect, balancing with the more synchronous inhibition it produces to retain the previous rate of discharge. The only effect of a tendon tap when the jerk is completely occluded is the transient synchronism in discharge. The decline of a tendon jerk at low passive tension reveals that active tension, however set in action, repercusses a central inhibition which is more than equivalent to its central exciting effect, and some subsidiary excitation from another source (from tension upon inactive units in a stretch reflex) is necessary to maintain discharge at that tension.

So far no means has been devised for isolating muscle-spindle from tendon-organs, for the latter, as Hinsey (29a) points out, are located among the muscle fibres reaching the aponeuroses of origin and insertion and not in tendon devoid of muscle substance. Their destruction therefore, as does destruction of the spindles, involves destruction of the muscle. The nearest approach to their isolation is the experiment of cutting down the ventral roots and recording from minimal units, and here it was found that the discharge was in general

more rapid and much more irregular, suffering cessation following the activity of certain units (? involving muscle-spindles).

The gravity response of pressure on the foot is described by Magnus (37) as the "positive supporting reaction," resulting in increased tone in the extensors. Such pressure on the pad of the foot is found myographically markedly to facilitate the extensors, and an instance of this is a case where such pressure, which by itself caused a slight excitation of 50 gm. (text-fig. 6) in soleus, combined with a stretch reflex of 450 gm. when alone, enhanced the

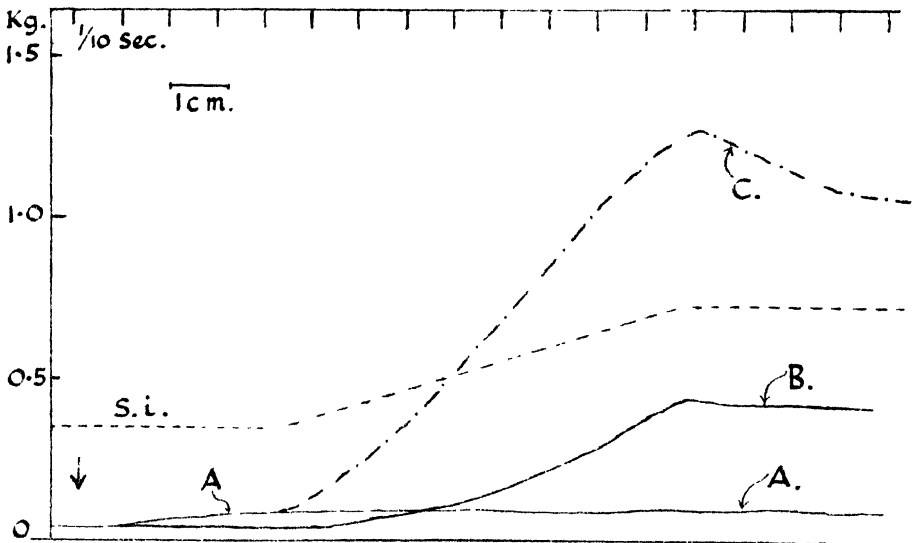


FIG. 6.—M. soleus. Decerebrate preparation (intercollicular section). AA—The maximal rise in tension produced by applying pressure to the pad of the foot (dorsum of the foot denervated). Greater pressure caused inhibition, less pressure less tension rise. The galvanometer string shows a few irregular action-currents. B—The effect of a stretch of some 4 mm. applied to the muscle. Broken line (*s.i.*) is the curve traced by the stretch indicator (table height). C—The effect of combining A and B.

latter to a peak of 1250 gm., maintained at 1100 gm., when the two were combined (intercollicular section). The reaction is a facilitation similar to that of the neck reflex, it plays upon the red muscles in greatest degree, and its effect without the stretch reflex is negligible.

A rapid passive shortening caused during the reflex of crossed extension will result, if the reflex be slight, in a complete suppression of discharge during the period of fall of tension (fig. 33). If the background be a powerful crossed excitation then very little effect is observed, while intermediate grades show an abolition of secondary waves, leaving often the stimulus rhythm well marked.

Crossed excitation, though its fraction varies in some degree with the crossed nerve chosen for stimulation (Eccles and Granit (18)), yet in its sustained recruiting phase plays within the maximal stretch-reflex fraction. Gastrocnemius and rectus femoris for instance have but very small fractions for crossed extension, while the large fractions are found in soleus and crureus and in lesser proportion in vastus internus and externus. This overlap of effect of crossed extensor within stretch reflex is well illustrated by the facilitations of the latter by the former obtained by Liddell and Olmstead (30a). The function of this reflex is certainly a mixed one, and in the intact animal its recruiting and sustained phase appears to act in enhancing the standing posture of the crossed leg, either during the flexion of the ipsilateral leg during a flexion produced by nociceptive stimulus, or by the act of stepping. Crossed extension is therefore essentially a powerful postural facilitation and its appearance in the de-afferent preparation is an expression of its potency in this connection, without necessitating the consideration of it as a posture without the stretch reflex. Like the other effects in the de-afferented limb it is then useless to the animal, not only in lack of direction but, what is more important, in lack of power and maintenance.

Reflex stepping likewise involves only the units in the fraction for the stretch reflex, and its discharge mechanism can be clonic or tonic, and reveals by analysis a slow rate of discharge. It consists, in the decerebrate and spinal animals, of a rhythmical waxing and waning of reflex standing, which is interpreted as the rhythmical appearance of a supplementary excitation over the whole stretch reflex field.

6.—*Concluding Remarks.*

The mechanism of the stretch reflex, thus outlined, accounts for the slow rate of discharge occurring in this reflex, for as shown in figs. 15 and 18 graded inhibition can slow the discharge of the de-afferented unit. This slow rate of discharge accounts for the relative lack of fatigue of decerebrate rigidity compared with a motor tetanus in the same muscle, the occurrence of its major portion in "red" muscle further explains the great difficulty in fatiguing these slight degrees of the reflex. The mechanism of red muscle will be discussed elsewhere, but it can be mentioned in this connection that observation of the surface of soleus with a microscope (objectives 2/3 inch and 1/6 inch) and with a powerful oblique lighting, shows that the blood flow in all vessels seen, both on the surface and for an appreciable distance under the surface of the muscle, is increased beyond all measure by elicitation of a stretch reflex, and no evidence

of retardation has been observed. Rarely a single muscle fibre or group of muscle fibres can be observed in partially fused contraction at a slow rate when the surface is fresh and bathed in warm saline and the muscle subjected to slight stretch. Rapidly contracting muscle fibre certainly takes part in the stretch reflex (*i.e.*, the response in pale muscles is not due to some intermingled red elements) as is evidenced by the rapid tendon jerk and the very rapid relaxation of the stretch reflex in pale muscles when inhibited. The slowly contracting element is therefore not essential and its employment as the focus of the reflex in the extensors is presumably linked with its resistance to fatigue, although it must be remarked that even rapid muscle is very difficult to fatigue in partial contractions with full blood supply.

The sympathetic nervous system is found not essential to any of the stretch mechanism here described, and the slight effects on general posture described by some authors (17, 40) must be explained by the possible existence of some subtle general effect on muscle contraction such as described by Obreli and his co-workers (11), or by an effect on the muscle-spindle which anatomically receives sympathetic innervation (8). No such effect has been discovered by excision or stimulation of the sympathetic, and the "silent period" is unaltered by these procedures.

The stretch reflex and its variations such as stepping (progression), crossed extension, and clonus, are essentially spinal in mechanism and occur in their usual form in the chronic spinal animal. Their enhancement by the labyrinthine and neck reflexes is seemingly differentiated for flexors and extensors and there is some evidence to show that it is reciprocal in effect. The effects of removal of various portions of the brain stem is a different matter and in the present state of knowledge of the working of these levels they indicate only that from the cerebral cortex down to the red nucleus, and especially in the region of the latter (10a), the *prevailing* influence upon the spinal mechanism is an inhibitory one. The inhibition obtained from the cerebral cortex has a widespread general effect on the spinal units, and the neurones of its departure from the cortical level certainly pervade the motor area (Cooper and Denny-Brown (13)) in a manner which lacks any simple reciprocal arrangement with the excitatory units which pervade the same cortical point. The removal of the cerebral cortex and of lower centres similarly microscopically varied in function, therefore, will affect the spinal motor units in some opposite degree, namely, a general excitatory effect according to the preponderating sign of the sum total of the systems removed.

A section of the brain stem likewise reveals by its effects only a general

release or general depression according to the balance of effects from above its level, and such evidence of flexor stretch reflexes as has been found tends to show that the release from mid-brain structure by intercollicular section and the depression of spinal shock, respectively promote and remove a subsidiary continuous excitation of all spinal motor units, flexor and extensor alike. The predominantly extensor effect of decerebrate rigidity is but the expression of the existence in the spinal cord of relatively large fractions of extensor units which are of low threshold for their proprioceptive afferent excitation. Thus the antigravity limb reflex is the result of a spinal functional arrangement.

The postural reflexes are therefore motor discharges of slow rate based upon a local spinal mechanism for each muscle, the stretch reflex of Liddell and Sherrington. There is no evidence that any plastic fixing mechanism occurs other than the conventional contraction process of muscle. The recruitment which is typical of their mode of development is evident not only as a fibre to fibre recruitment, but coarsely as an involvement of a muscle head by head, and is likewise the result of a spinal arrangement, the gradients of central excitability of neurones.

Summary of Conclusions.

1. The mechanical phenomena of the postural reflexes can be fully accounted for by motor excitation of the muscles concerned, without the addition of any plastic fixation other than that of the conventional contraction process resulting from that excitation.
2. The motor discharge in such postural reflexes is a relatively slow repetitive impulse series, thus accounting for their relative resistance to fatigue.
3. Central excitation is a process of summation, even with seemingly direct effects, and the motor response to that summation is a rhythmic series of waves of excitation discharged by the motor unit. The rate of these waves is the outcome of the relative amounts of inhibition and excitation summated at that unit.
4. The slow regular motor discharge of posture is the result of a central excitation, in which proprioceptive excitation and proprioceptive inhibition are summed with a subsidiary adjuvant excitation, part of which is derived from higher levels of the nervous system. Under especial conditions the central summation itself becomes rhythmic, resulting in the phenomenon of clonus.
5. In accordance with their threshold of response to such central summation of proprioceptive effects the motor units of a muscle can be graded. The

units of lowest threshold grade are present in the red slow extensors and are responsible for the slow tendon jerk when central excitability is low, as in the condition of spinal shock. The units of higher threshold grades, according to their grade, require more subsidiary excitation before responding to proprioceptive excitation by discharge. These units are arranged evenly through the different heads of the compound extensors so that the units of highest threshold are confined almost exclusively to the pale muscles.

6. The flexor centre possesses a few units corresponding to the extensor units of moderate grade of threshold, and many units which correspond to the extensor grades of highest threshold.

7. The proprioceptive reflex resulting from this mechanism, the stretch reflex of Liddell and Sherrington, is subject to facilitations by other reflexes and for some of these the effects are powerful enough to cause discharge without the stretch reflex, resulting in the low discharges of partially maintained type in the de-afferented extensor muscle.

8. The onset of passive stretch by momentarily causing greater central excitation without a corresponding degree of inhibition, causes a transient excess of discharge, which in its turn causes an inhibitory reflex which again establishes equilibrium. This reaction during or immediately after the increase of stretch is the "lengthening reaction." Release of stretch similarly destroys the equilibrium in favour of the inhibition, recovery of the tension excitation causing the "shortening reaction."

9. Active tension alone reflects to the centre more proprioceptive inhibition than is equivalent to the proprioceptive tension excitation it alone can cause, and in the absence of central excitation of other origin such active tension (*e.g.*, as is produced by a tendon jerk) declines until sufficient passive tension on inactive units is added to bring the discharge to equilibrium.

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DESCRIPTION OF PLATES.

All figures read from left to right. In the myograph records of mechanical contraction an upward deflection indicates a rise of tension. Time is indicated at the top of each record by vertical strokes at 1/50 second intervals, produced by the shadows of spokes of a Rayleigh wheel (each 1/10 second is stressed as a thicker stroke). The action-currents are recorded by a string galvanometer of Cambridge pattern with silvered quartz strings varying from $1.3\ \mu$ to $2.2\ \mu$ in thickness (magnification $\times 390$). Where two action-current records are recorded concurrently a double string case is used with two paired strings, each approximately $2.3\ \mu$ thickness, with an entirely separate circuit for each. The leads are two silver pins, coated electrolytically with a thin layer of chloride, both in the muscle substance. With careful insertion of such thin pins between the fasciculi of the muscle, after first cleaning off the perimysium, no injury current results, and no compensation is therefore necessary. All preparations (including spinal dogs) were decerebrated under deep anæsthesia and left for over 3 hours before recording, to allow recovery from the effects of the anæsthetic. The animal used is the cat unless otherwise stated. As in earlier papers the signal for the stimulus is the vertical movement of a shadow of the key which un-shortcircuits and re-shortcircuits the secondary circuit in the case of series of stimuli, and the shadow of the key which breaks the primary circuit in the case of single break-shock stimulus. The series of break-shocks at regular rate are obtained continuously from a Sherrington torsion-wire key. Muscle stretches are given by a fall-table as used by Liddell and Sherrington (31).

PLATE 11.

FIG. 1.—M. Soleus. Decerebrate preparation. A stretch reflex totally inhibited by a faradic stimulus at 50 break-shocks a second, beginning at the fall of the first signal, to the central portion of the cut nerve to the ipsilateral hamstring muscles (hamstring

nerve). This stimulus is again short-circuited by the fall of the upper signal, but the inhibition remains complete for some time afterwards, allowing full relaxation. The cessation of action-currents is immediate and complete. Tension scale as in fig. 22.

FIG. 2.—M. Soleus. Decerebrate preparation. An excitation of the crossed sciatic nerve begins with the fall of the lower thick signal line, and a stimulus to the posterior tibial nerve of the same side, with the rise of the upper signal line. In dotted lines is superimposed the tension curve of a short motor tetanus produced by 4 break-shocks at a rate of 50 a second. Twitch duration 195 σ . The cessation of action-currents indicates the complete inhibition. Tension scale as in fig. 25.

FIG. 3.—M. Soleus. Decerebrate preparation. Single motor unit in discharge as evidenced by the action-currents, with later stretch applied. The thick rising line is the myograph shadow, the thin rising line is the shadow of the stretch indicator. Tension scale as in fig. 22.

FIG. 4.—A—The same preparation as fig. 3, the muscle is subjected to a slight unvarying stretch. B—As in A.

FIG. 5.—M. Soleus. Decerebrate preparation. A single weak break-shock is applied to the hamstring nerve of the same side at the fall of the thick signal. A single unit misses a beat, but rapidly recovers after a preliminary acceleration.

FIG. 6.—M. Soleus. Decerebrate preparation. A stretch of the muscle is indicated by the stepped rise of the stretch-indicator (thin sharp line). The action-currents are obtained concurrently in two galvanometer strings, A and B, by a set of leads in the distal portion of the muscle for A, and another set in the proximal portion for B. Large animal (5 kilo.) and large muscle. The different units may be identified by shape and size, and the rhythms of four units are indicated. The unit in the upper string (x) is followed easily and not interfered with. Units a , b and y are all seen as very faint deflections in the upper string, where their shape assists in unravelling their sequence when they overlap with others in the lower string. Two distinguishable units, besides a , b , x and y , make late appearances, discharging rhythmically. Tension scale as in fig. 32.

FIG. 7.—M. Soleus. Decerebrate preparation. A stretch of soleus, applied so slowly that only one unit is "recruited" during this period. Tension scale as in fig. 32.

FIG. 8.—M. Soleus. Decerebrate preparation. Gradual removal of the stretch. Three units, a , b and c being at first involved, with first c and then b ceasing contraction abruptly. Tension scale as in fig. 32.

PLATE 12.

FIG. 9.—M. Soleus. Decerebrate preparation. One unit in rhythmic discharge and interrupted by a light tendon tap (sharp mechanical deflection), which causes a tendon jerk. Following the large wave of the jerk (a single wave in many units) the original unit, after a silent period, begins beating at an accelerated rate which is maintained until the end of the plate. Tension scale as in fig. 32.

FIG. 10.—M. Soleus. Decerebrate preparation. No units discharging at first. A weak break-shock to the posterior tibial nerve of the same side causes the appearance of "rebound" as the beating of two units, a and b . The unit b beats only twice, while a continues for the whole of the record, gradually slowing. The fall of the second

signal indicates only the short-circuiting of the secondary, preparatory to setting the keys for the next break-shock. Tension scale as in fig. 32. Tension rise less than 10 gm.

FIG. 11.—*M. Soleus*. Decerebrate preparation. Double set of leads and galvanometer strings. String A from proximal leads, B from distal leads. Muscle under slight stretch. A weak tetanic stimulus (rate 50 a sec.) is applied to the ipsilateral hamstring nerve at the fall of the first signal and ceases with the fall of the second signal. Unit *c* is slightly accelerated by this stimulus and its after-effect (reflex of ipsilateral excitation), while units *a* and *b* cease discharge after two beats in *a* and one in *b*, and return after cessation of the stimulus accelerated in rate. Tension scale as fig. 32. Tension fall about 10 gm.

FIG. 12.—*M. Soleus*. Decerebrate preparation. Ventral root 1 S., and all dorsal roots, intact, remainder of ventral root supply sectioned. In A, a stretch of muscle brings into activity three units, *a*, *b* and *c* in A. Unit *c* inhibits units *a* and *b*. In B, a smaller stretch involves only unit *a*, which beats rhythmically when alone. Tension scale as in fig. 32. Time in B as in A.

FIG. 13.—*M. Soleus*. Decerebrate preparation. Ventral roots 6 L, 1 and 2 S, sectioned and 7 L intact. Dorsal roots intact. Stimulus to the crossed sciatic nerve. The discharge commences with the rapid sequence of a small wave, followed by the slower sequence of a large wave which is not accompanied by the small wave. After three beats of the large wave the small rapid wave reappears.

FIG. 14.—*M. Soleus*. Same preparation as for fig. 13. During an excitation from the crossed sciatic the muscle is suddenly stretched (beginning at the arrow). More numerous waves at a rapid rate follow this procedure. Tension scale as in fig. 32.

FIG. 15.—*M. Soleus*. Decerebrate preparation. Completely de-afferented and almost completely de-efferented by section of the 6 and 7 L, and 1, 2 and 3 S dorsal roots, and the 6 L, 1, 2 and 3 S ventral roots, with the 7th L ventral root cut down until only one small fasciculus remained. A regular repetitive stimulus at a rate of 50 break-shocks a second to the anterior femoral nerve of the opposite side is continued all through. At the fall of the upper signal a regular series of break-shocks at 65 a second is applied to the ipsilateral internal saphenous nerve until the fall of the second signal. Fall in tension of some 12 gm. due to cessation of other units.

FIG. 16.—*M. Soleus*. Also completely de-afferented, and with only partial motor innervation as in fig. 15, from another preparation a stimulus to the anterior femoral nerve of the opposite side begins with the fall of the first signal and ceases with the fall of the second. Rate, 50 a second. Tension rise 10 gm.

FIG. 17.—*M. Soleus*. As fig. 16, more rapid record.

PLATE 13.

FIG. 18.—*M. Soleus*. As figs. 15 and 16, with an inhibitory stimulus to the ipsilateral anterior femoral nerve. (Excitatory rate 50 a second, inhibitory rate 65 a second, as in fig. 15, from another preparation.) The after-effect of the inhibition is much shorter than in fig. 15. No alteration of tension (scale as fig. 32).

FIG. 19.—*M. Soleus*. As in fig. 16, excited by a very weak stimulus to the anterior femoral nerve of the crossed side. Note double waves, and after-discharge rate (the same as before, although stimulus ceases when the signal drops).

FIG. 20.—M. Soleus. Decerebrate preparation. All nerve supply intact. Minimal stretch response in one unit. An exceedingly light tap, indicated by the peak in the myograph record, causes an early response in the beating unit, but otherwise affects it but little. Probably only one other unit is caused to discharge by the tap, and then but once. Tension scale as fig. 32.

FIG. 21.—M. Vastus Internus. Decerebrate preparation. De-afferented and all ventral roots sectioned except for a portion of the 5th lumbar root. The termination of a stimulus to the anterior femoral nerve of the opposite side occurs at the fall of the signal. A unit with an action current composed of two downward deflections in rapid sequence (*a, a*) can be traced back from the after-discharge into the discharge during the stimulation period. The fall of tension of grams is greater than is produced by the units shown in discharge here, owing to the size of the muscle head and the localisation of the galvanometer leads. Tension scale as in fig. 32.

FIG. 22.—M. Quadriceps. Decerebrate preparation. A stretch reflex becomes clonic in type. Stretch indicator shows the period of application of stretch and its maintenance. Note varying synchronism of mechanical beats and accompanying action-currents, especially the phase of greater synchronism commencing at the end of the record.

FIG. 23.—M. Quadriceps. Decerebrate preparation. An ill-defined clonus has been produced by a previous slight stretch and a tendon jerk with clonic after-discharge is elicited. Note the asynchronous third beat in the jerk and the recovery of greater synchronism in the ensuing beat. Tension scale as in fig. 25.

FIG. 24.—M. Quadriceps. Decerebrate preparation. Maintained stretch reflex of clonic type showing variation in synchronism. Tension scale as in fig. 25.

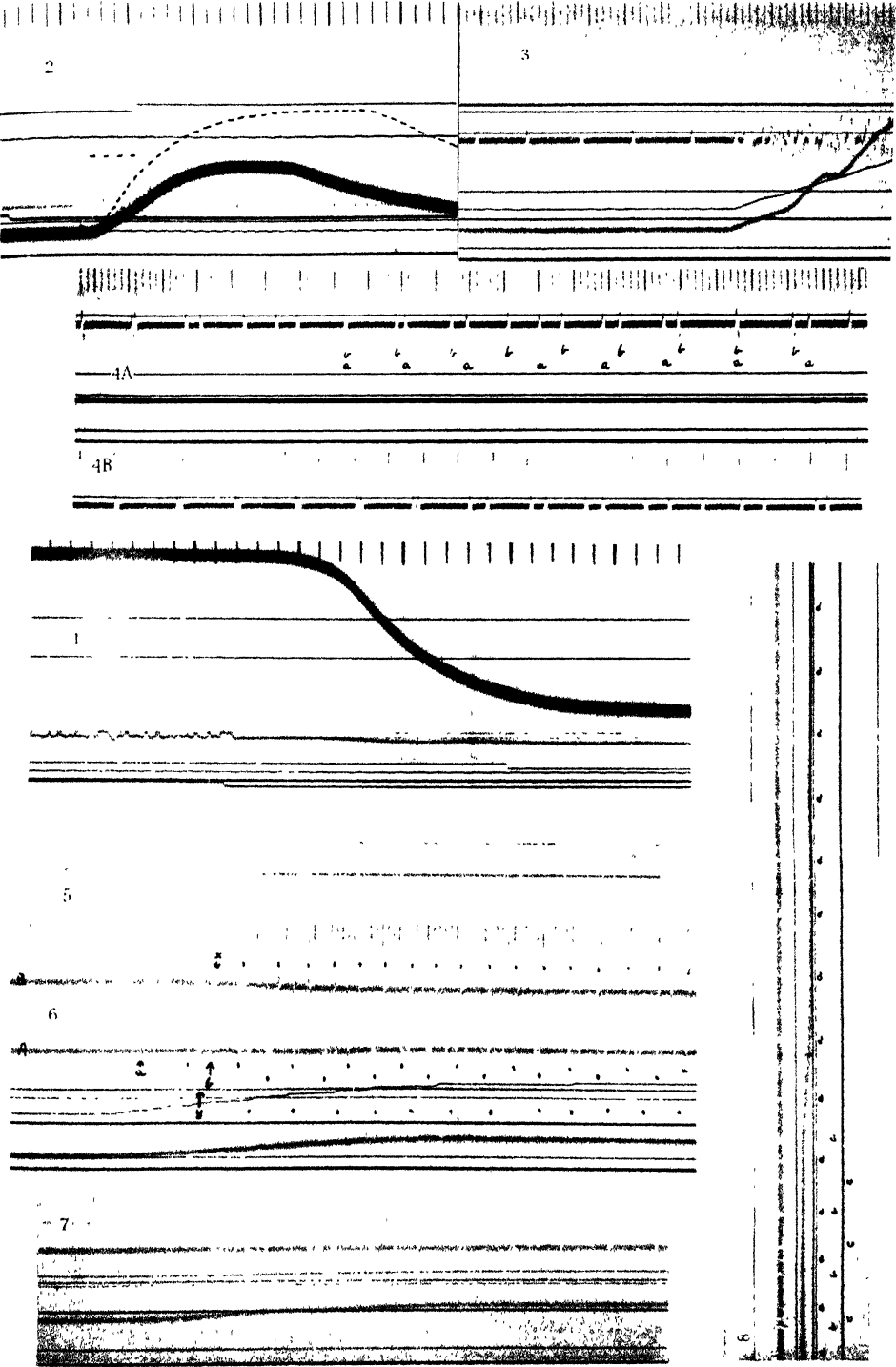
FIG. 25.—M. Quadriceps. Decerebrate preparation. Maintained stretch reflex of clonic type. Change of rhythm caused by the vibration from a tap to the myograph table at the arrow. A short steel spring here intervenes between the muscle hook and myograph lever, so that greater shortening is allowed for each contraction and the larger mechanical waves are damped. The mechanical record therefore is here true for the average tension but not for the maximum and minimum of each beat.

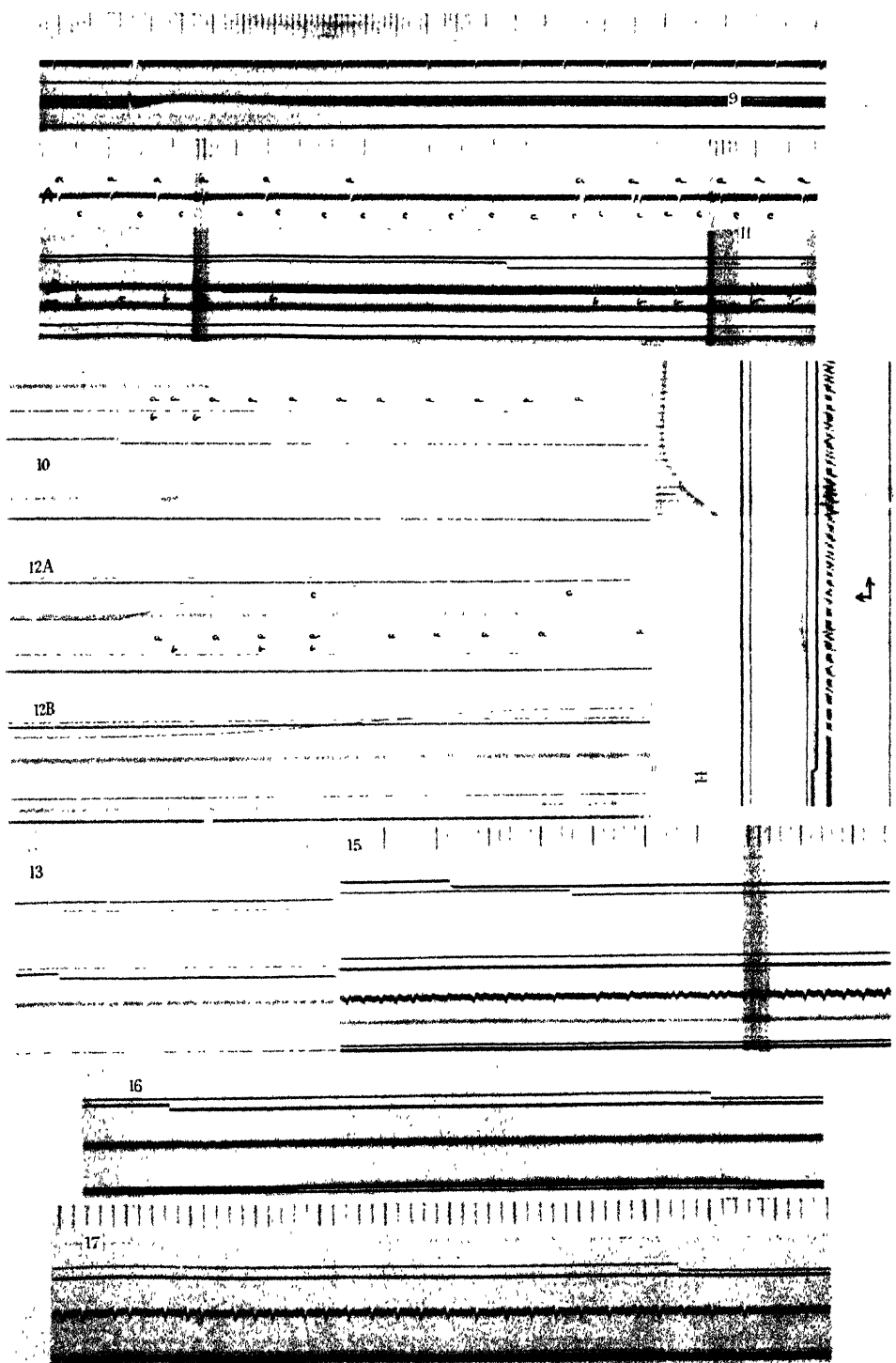
PLATE 14.

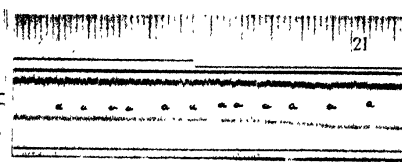
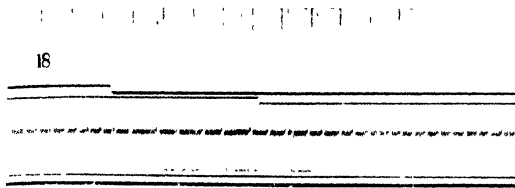
FIG. 26.—M. Gastrocnemius. From a dog with complete transection of the spinal cord behind the 12th thoracic segment 11 months and 10 days earlier. Previous slight stretch reflex with tendon jerks. Tension scale as in fig. 27.

FIG. 27.—M. Gastrocnemius. From the same preparation as in fig. 26. Stretch reflex and action-currents.

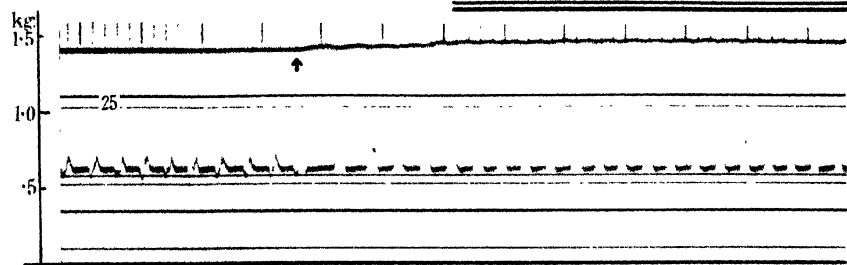
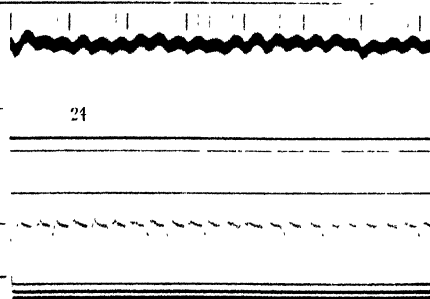
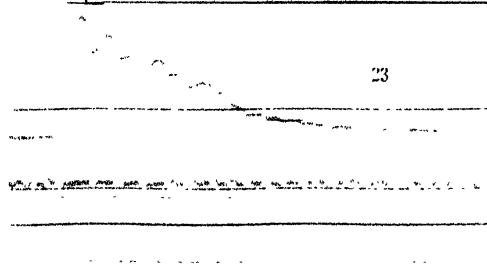
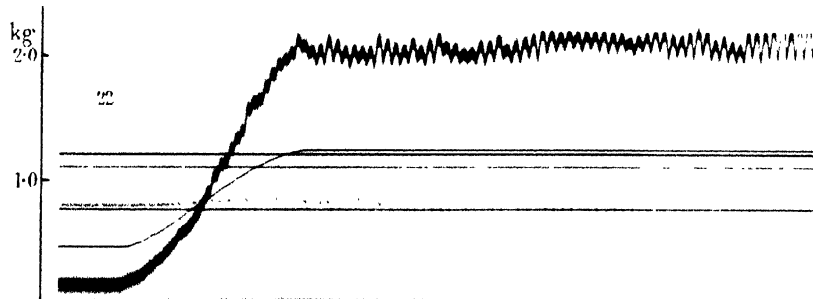
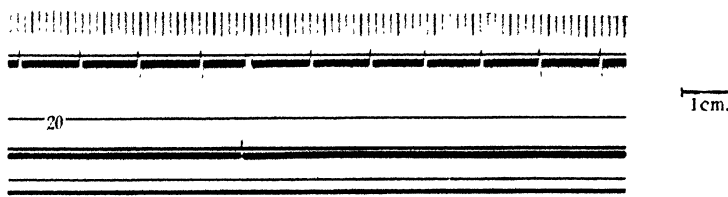
FIG. 28.—M. Quadriceps and M. Soleus. Decerebrate preparation. The lower galvanometer string records the action-currents in quadriceps which is clonic in response to stretch. The upper string records the action currents in soleus, which is thrown into fused mechanical contraction by the elicitation of clonus in quadriceps. In general the rhythm of soleus follows that of quadriceps, but at *a* and *b* it breaks down to asynchronous beats of longer interval, later again showing the rhythm of quadriceps. The beats, when "in time" in both, are in fact between 2 and 3 σ late in soleus.







19



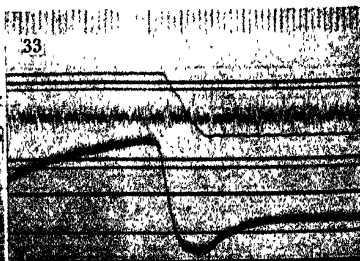
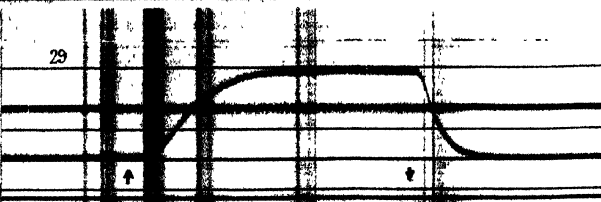
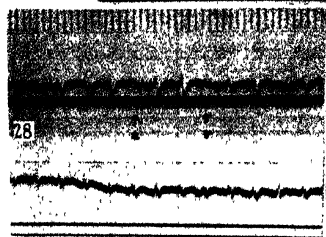
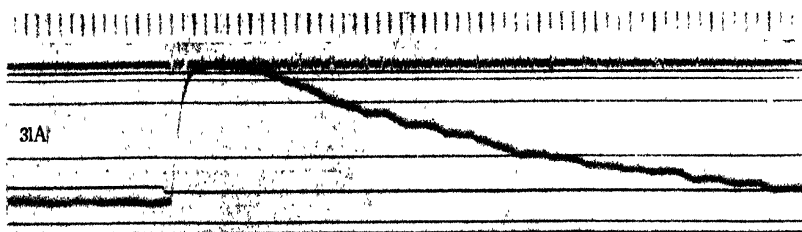
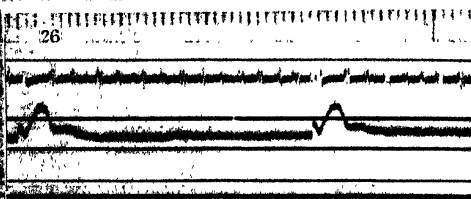
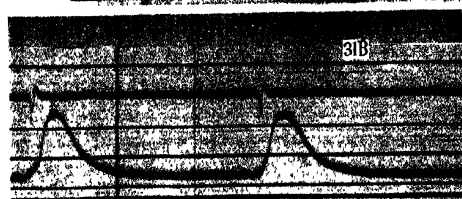
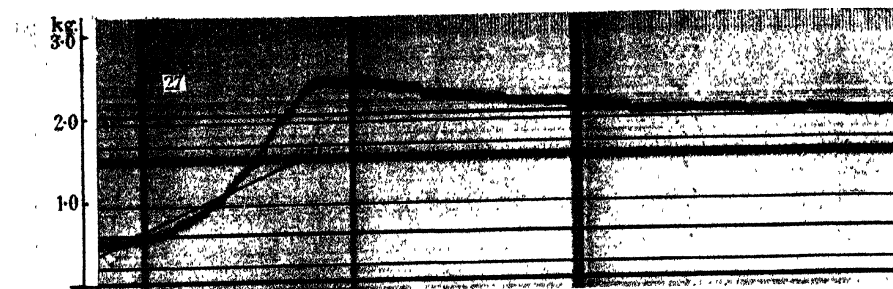


FIG. 29.—*M. Soleus* (left). Decerebrate preparation. Completely de-afferented. Contraction caused by turning head to the left at the arrow (↑) and maintaining it so until the second arrow (↓) when the head was turned to the right. Since the animal was lying on its right side the labyrinth was approximately respectively maximal and minimal in these positions and thus was assisting the effect of the neck reflexes. Tension scale as in fig. 25.

FIG. 30.—*M. Tibialis Anticus*. Decerebrate preparation. A tap upon the tendon every 0·5 second elicits when alone a very small tendon jerk response, but when combined with a stimulus of 50 break-shocks a second to the sciatic nerve of the opposite side, commencing at the fall of the first signal (indicated by (↑)), and ceasing at the fall of the second signal (indicated by (↓)) the jerk response is greatly enhanced, although the contraction caused by the nerve stimulus, as seen between the jerks, is very slight. The after-effect of the stimulus is also revealed by the enhancement of a jerk 0·5 second after cessation of the stimulus.

FIG. 31.—*A*—*Tibialis Anticus*. Spinal dog, section at 12th thoracic level over 11 months earlier, another preparation similar to that used for figs. 26 and 27. At the fall of the signal a single break-shock is delivered to the posterior tibial nerve of the same side. Note the postural rhythm in the galvanometer string before the break-shock, and the long-maintained after-discharge of the contraction, part of which is almost certainly facilitation of a subliminal fringe of stretch response by the break-shock excitation. *Gastrocnemius* retains its innervation, but is slackened.

B.—*Tibialis Anticus*. Decerebrate cat. Tendon jerks with a background rhythm of fine asynchronous action currents caused by stretch. *Soleus* retains its innervation, but is slackened. Tension scale as in fig. 30.

FIG. 32.—*M. Soleus*. Decerebrate preparation. Stretch reflex caused by gradual passive lengthening of the muscle (gradual rise of stretch indicator) and the cessation of discharge, with some later recovery, caused by sudden passive shortening of the muscle (sudden fall of stretch indicator).

FIG. 33.—*M. Soleus*. Decerebrate preparation. Excitation by stimulus to the crossed posterior tibial nerve, at a rate of 65 break-shocks a second, continues throughout. At fall of stretch indicator the muscle is passively rapidly shortened, with resulting diminution of the number of action-currents in the discharge. Tension scale as in fig. 25.

*Study of Golgi Apparatus and Vacuolar System of Cavia. Helix,
and Abrazas, by Intra-Vital Methods.*

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(Communicated by E. S. Goodrich, F.R.S. -- Received October 15, 1928.)

[PLATES 15-16.]

Introduction.

Up to a few years ago, cytologists in general had accepted the view that the animal cell consisted of a nucleus and a cytoplasm containing granulations of two-types, Golgi bodies which had the power of forming vesicles, and mitochondria or chondriosomes. Most people have been reluctant to admit the presence of a third cytoplasmic constituent, apart from the centrosome.

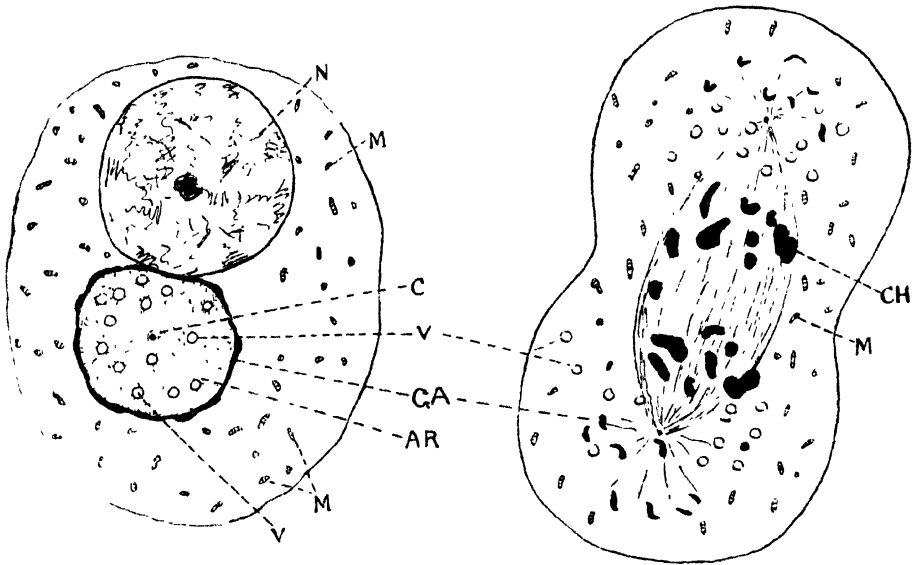
Recently Parat and his school of workers have claimed that the cell has a system of vacuoles, containing a substance stainable especially in neutral-red, and that this system is the homologue of the Golgi apparatus of nerve cells, first demonstrated by Camillo Golgi.

For many years botanical cytologists have recognised a vacuolar system in plant cells, and Parat and Guilliermond have sought to homologise the vacuolar system in plants with Parat's "vacuome" in animal cells, and by inference to identify the vacuolar system of plants with the Golgi-canalicular system of nerve cells. Parat's views, and those of the writer, are shown in text-fig. 1. According to Parat the dark cortex of the archoplasm (AR) is the lepidosome system of modified mitochondria (GA), the real Golgi apparatus being the vacuolar system (V).

The writer's view, which appears to be shared by most other cytologists, is that the dark cortex is the homologue of the argentophil or osmiophil substance of the nerve cell. The neutral red-staining vacuoles, when present, are not argentophil or osmiophil, but are often closely related to the true Golgi apparatus, possibly derived from the latter.

Parat, at the time of the launching of his vacuome hypothesis, received little support outside France, and his view that the vacuome is the Golgi apparatus is disproved by one fact alone: *the neutral red-staining vacuome is not consistently argentophil whereas the Golgi apparatus is.* The writer knows no case

in animals where the vacuome is argentophil: in plants, the vacuoles may, as in the case of the bean root tip cells, be argentophil.*



TEXT-FIG. 1.--Scheme of resting and dividing cell. N, nucleus. M, mitochondria. C, centrosome. V, vacuolar system. GA, Golgi apparatus. AR, archoplasm. CH, chromosome. (Based mainly on molluscan type.)

Recently the writer has seen the following résumé of a paper (to appear in the 'Anatomical Record') which was read (1928) at Ann Arbor, before the American Anatomical Society, by W. P. Covell and G. H. Scott:—

"Smear preparations of ventral-horn cells and of spinal-ganglion cells of white mice and young rabbits, vitally stained with neutral-red, were treated with osmic acid and with the silver-impregnation methods for demonstration of the Golgi apparatus. Individual cells were observed throughout the various steps in the treatment. It was possible to follow through the actual process of the osmication and silver impregnation of individual granules which had previously been stained with neutral-red. The findings support the hypothesis advanced by Parat and his co-workers that the Golgi apparatus results from the treatment of neutral-red-stainable granules with silver and osmic acid."

* It is doubtful if the vacuoles of plant cells are the same thing as the animal neutral-red vacuoles.

Now examination of such neurones as that of *Helix* shows that the "Golgi apparatus" consists of scattered isolated elements, each of which is formed of an argentophil or osmiophil dictyosome (homologous with the mammalian argentophil Golgi apparatus) *plus a chromiophile part, not necessarily associated with a globule of neutral-red-staining substance.* In the case of the mammal the



TEXT-FIG. 2.—Neurones of Cat, dorsal root ganglion. Golgi apparatus (G), vacuolar system (Holmgren) (V).

real Golgi apparatus is a very thin pellicle (G) on the canal or vacuole (V) (text-fig. 2), and the latter never shows properly with the silver Golgi methods. With these methods it almost completely collapses and does not stain. Covell and Scott have seen the neutral-red part, have impregnated its cortex afterwards, and have concluded that the neutral-red part is the same material as goes black with silver, and as is usually known as the Golgi apparatus. Camillo

Golgi never saw the neutral-red associate of the Golgi apparatus, so far as the writer can discover from his publications.

In his work on plant cells Bowen (2) has finally exploded the hypothesis of Guilliermond* and Dangeard with reference to the nature of the vacuolar (so-called Golgi canals) in plants, by the discovery of true Golgi elements in many plants. These platelets are the subject of a forthcoming paper by the writer and two of his students, and are considered as the undoubted homologues of the Golgi bodies of animal cells.

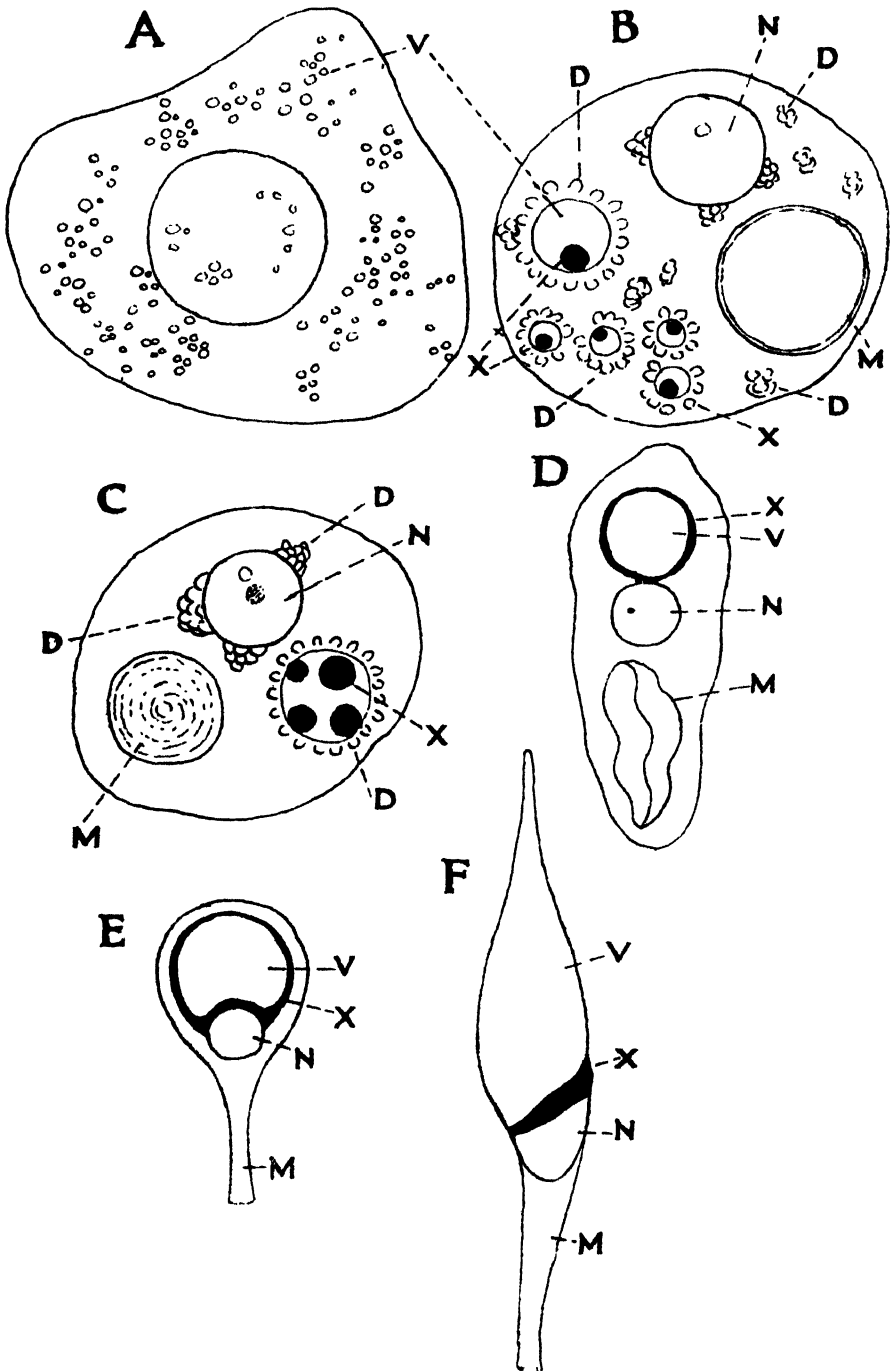
Bowen's discovery of true dictyosomes in plants, which has been confirmed in this laboratory by the writer's students Miss Patten and Miss Scott, has clarified an obscure part of plant cytology, and may be considered a first-class contribution to our knowledge of plant cytology. The writer's own investigations, together with the important work of Voinov, Hirschler and Monné, has convinced him that the vacuolar system in the male cells may be separated from the Golgi bodies themselves, though in the case of the egg and gland cells, the latter bodies seem to be constantly associated with vacuoles, which may be the vacuolar system.

Previous Work.

It is not proposed to enter into the well-known work of Parat, Painlevé, and other workers of this school, but some of the results, more recently published, of Hirschler, Monné, Voinov, and Karpova will be dealt with here. One of the most remarkable accounts of the vacuolar system in spermatogenesis is that given by D. Voinov (14) for *Notonecta glauca*, the water-boatman.

In fig. 3, stage A, is the spermatocyte, which contains groups of red granules (V). In the early spermatid these granules run together to form large vacuoles, which, by stage B, have attracted to themselves many of the ordinary dictyosomes or Golgi bodies (D). Finally all the vacuoles run together to form one very large structure shown in stage C, at D, X. Before this happens, however even in the subsidiary vacuoles shown in stage B, there has appeared a darker structure (X) which stains deep red, and which, according to Voinov, is secreted by the dictyosomes. In stage C, the new substance (X) is in the form of four deep-red granules, inside a vacuole full of pink-staining liquid. The mitochondrial "nebenkern" is at M. In both stages, B and C, sticking on to the

* "Les recherches de Corti et surtout celles de Parat et Painlevé ont ensuite apporté une confirmation à cette opinion dans la cellule animale, et montré qu'effectivement les canalicules de Holmgren et l'appareil de Golgi correspondant à une même formation, colorable sur le vivant par le rouge neutre et semblable au vacuome de la cellule végétale" (Guilliermond, 'C.R. Acad. Sc.', vol. 182).



TEXT-FIG. 3.—Spermatogenesis of *Notonecta glauca*. (For explanation see text.)

nucleus at D, are other dictyosomes which did not pass on to the surface of the vacuome.

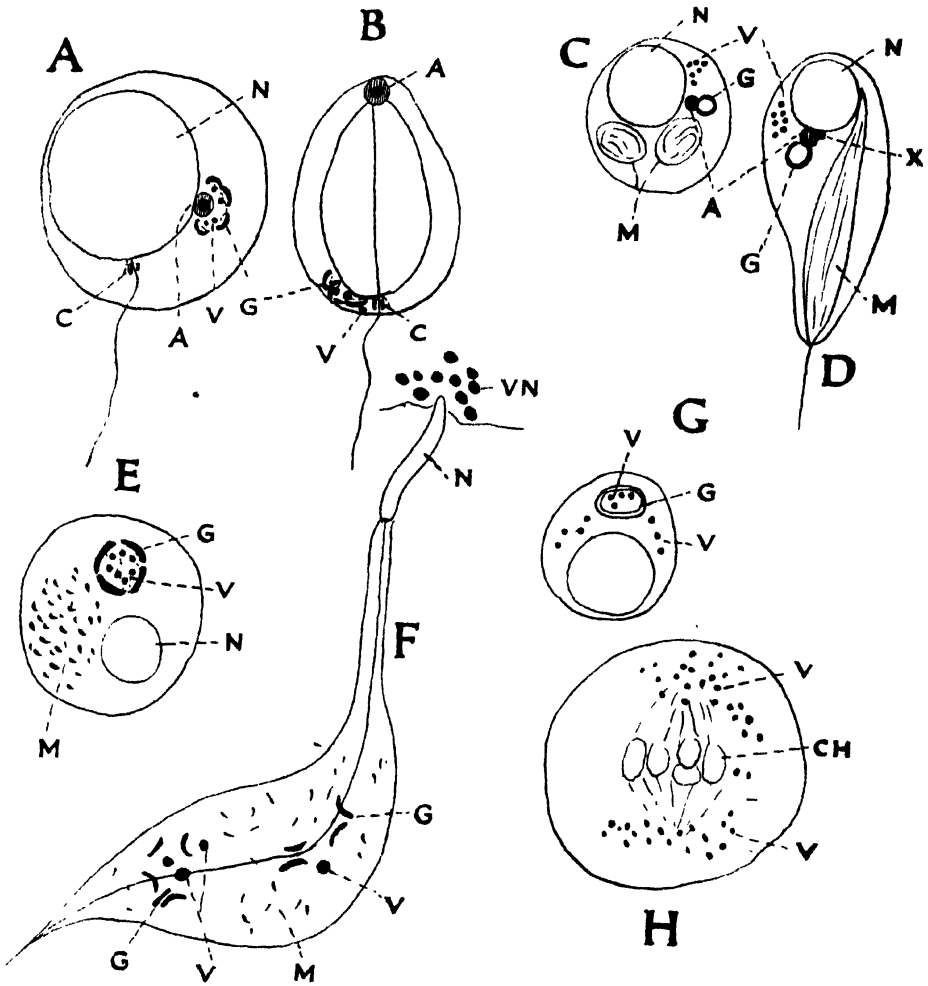
Voinov gives no explanation of the adherence of these dictyosomes to the nucleus, except to say that they may play a rôle in the exchanges between the nucleus and the cytoplasm. To those who have seen the spermatid dictyosomes forming the acrosome, while in contact with the nucleus, it seems strange to read that in *Notonecta* the acrosome is formed away from the nucleus by other but similar dictyosomes stuck upon the vacuome. Yet it must be admitted that Voinov's account and figures are convincing.

The subsequent stages are also depicted in text-fig. 3, D, E, and F. The new substance, X, ultimately forms that part of the acrosome abutting against the nucleus, the vacuolar substance itself forming the bulk of the pointed region of the acrosome, stage F, at V. This spermateleosis is unique, and in parts difficult to understand. It is at present the only spermatogenesis known in which the extra-archoplasmic vacuolar system passes into the ripe spermatozoon. Voinov has so carefully followed his intra-vital studies with sections of testes, fixed and stained by the most perfect cytological techniques known to us, that the writer has no hesitation in accepting this account.

In text-fig. 4 is a number of cells drawn after Hirschler (6, 7, 8, 9), Monné (11), and Lydia Karpova (10). The lettering on Mlle. Karpova's figs. E and F are the writer's, for this cytologist was a disciple of Parat. at the period this paper was written. Figs. A and B are lizard spermatids, showing the acrosome at A, the vacuoles at V, inside the archoplasm of the Golgi elements at G. In fig. 4, B, the acrosome is *in situ* on the head of the sperm, while the vacuoles and Golgi elements have drifted down to the base of the cell. The lizard is a type in which the vacuoles keep inside the archoplasm, until individual Golgi elements are somewhat scattered as spermateleosis proceeds.

Figs. 4, C and D, of a grasshopper, are also after Jan Hirschler, and show a "vacuome," (V), separate from the Golgi dictyosome (G). The latter secretes the acrosome at A, and the latter in fig. 4, D, is seen to have a redder granule (in neutral-red preparations) which is marked X. This type resembles the moths.

Figs. 4, E and F, after Mlle. Karpova, show the intra-archoplasmic vacuome, which had been overlooked by the chrome-osmic technicians. Mlle. Karpova would look upon the vacuoles (V) as the Golgi apparatus, and the dictyosomes, marked G, as the lepidosomes (modified chondriome) of Parat. In fig. 4, F, the Golgi bodies (G) have got disarranged, and the "vacuome" also has drifted out of position in the archoplasm.



TEXT-FIG. 4. - Spermatogenesis of Lizard (A and B), *Palomena* (C and D), *Helix* (E, F and H), and *Cerithium* (G). (For explanation see text.)

In figs. 4. G and H, are two interesting molluscan cells, after Hirschler's pupil Monné. Fig. 4. G, of *Cerithium*, shows a vacuolar system, partly inside the archoplasm, partly outside. In fig. 4. H, is a spermatocyte metaphase in *Helix*.

Technique.

Up to recent times the writer and his students have depended largely on chrome-osmium techniques. These, with iron alum-hæmatoxylin staining, and the newer Kolatchev and Mann-Kopsch methods, are the most perfect

cytological techniques known, but it is only in rare cases that they will show the vacuolar system well.* As will be mentioned below, such methods show the vacuolar system in the Archannelid, *Saccocirrus*, in which the writer was the first to describe the system in 1922 (4). But at that time the nature of these vacuoles was not understood.

Parat and his pupils have constantly used neutral-red solutions to reveal the vacuolar system. We know little, if anything, about the nature of neutral-red staining. It is said that neutral-red is a delicate indicator of acidity and alkalinity, from which we may assume for the time being that the vacuoles which become so intensely stained by weak solutions of neutral-red contain some acid substance.

The *modus operandi* is to dissect out the gonads, etc., of some animal, and place a small fragment of tissue in a drop of Ringer, coloured pink with neutral red. After 15 to 30 minutes the fragment of tissue is teased up in the drop of fluid, and a cover-slip is placed on the slide. In certain cases, *e.g.*, lepidopterous gonads, the vacuolar system is found to be stained straight away, but in molluscs the staining takes much longer. During this time the cells do not become altered or die. Provided that the Ringer and slides are sterile, the cells will live for many hours in an unaltered condition. The German worker Goldschmidt has watched lepidopterous spermatogenesis in such live cultures for long periods.

It usually takes at least 30 minutes for a pale pink Ringer solution properly to stain up the vacuolar system in any animals. Parat and his pupils have often fed neutral-red to animals, or have injected it into the abdominal cavity. Fresh-water aquatic larvae, etc., may be kept in tanks of water tinged with neutral-red. In the case of aquatic molluscs, we have not found this particularly successful, because even though the animals became pink or pale red right through, the germ cells failed to show the vacuolar system. This method was tried especially with species of *Limnaea*.

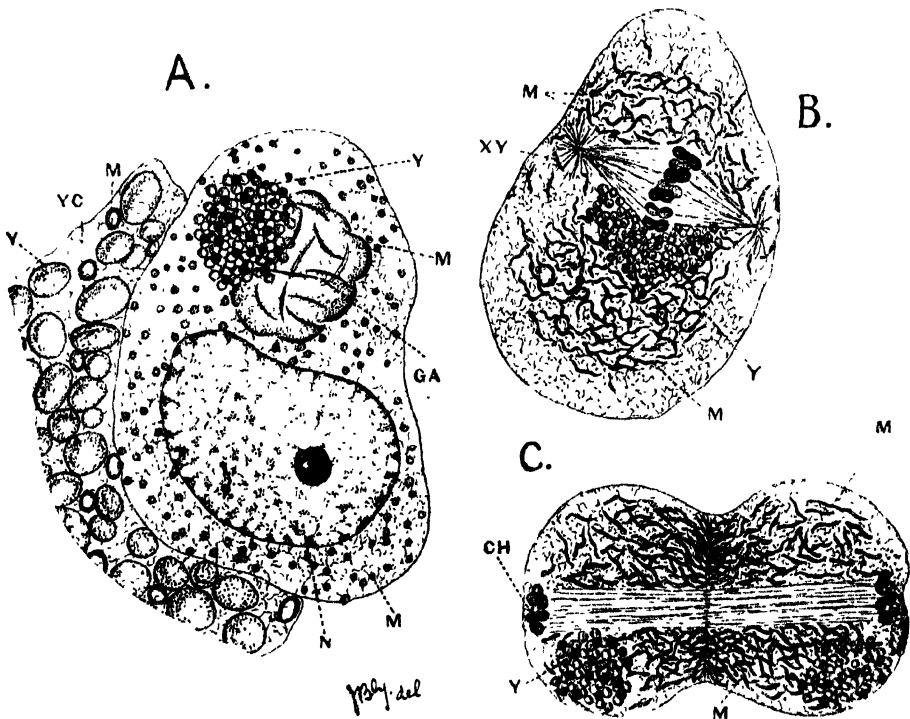
In general, it may be stated that such intra-vital observations with neutral-red are useless, unless the worker is thoroughly acquainted with the appearance of the same cells fixed by modern chrome-osmium techniques in a way to reveal the cytoplasmic inclusions. The worker so acquainted is able easily to find both Golgi bodies and mitochondria in the delicate living cells. The writer has studied by this intra-vital method a number of invertebrates, and the mammal, *Cavia cobaya*, as well as many Protozoa.

* Dr. Sylvia Wigoder and the writer find that Kolatchev preparations, counter-stained into Ludford's neutral-red, shows the guinea-pig vacuolar system.

Original Observations.

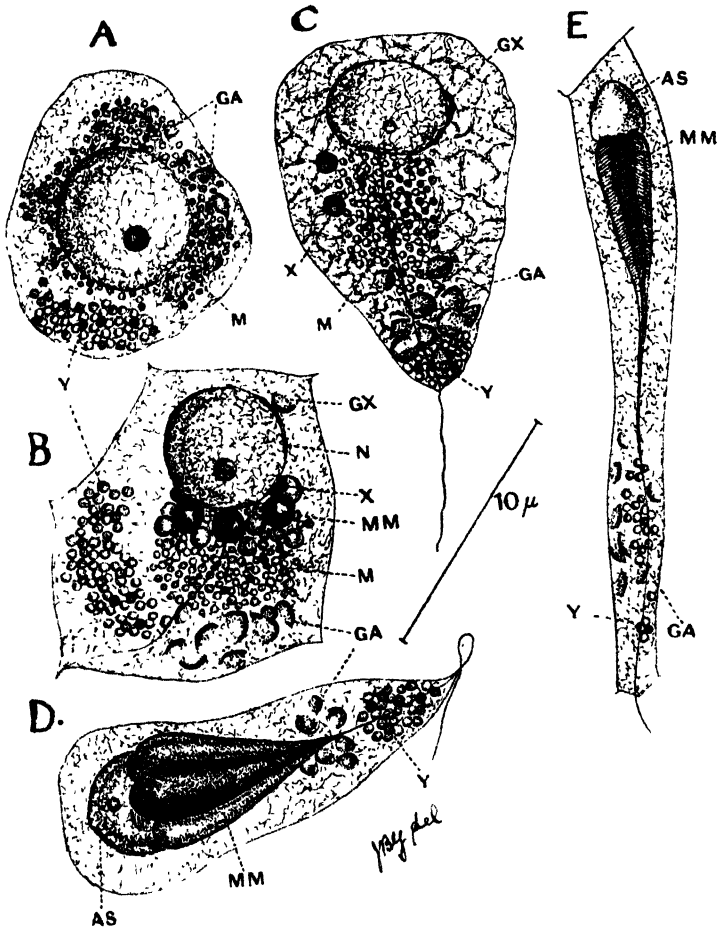
Abraxas grossulariata. In the growing spermatocyte at the time of formation of the four flagellar organs (*f*, in Plate 15, fig. 1) the neutral-red vacuoles occupy a position towards the lumen, and near the centrosomes. It has not been possible to find the vacuoles inside the Golgi apparatus, but it is likely that in the spermatogonium the vacuoles lie inside the Golgi apparatus, as shown in fig. 20, for the snail *Helix aspersa*. In the full-grown spermatocyte, the neutral-red vacuoles still tend to keep in a group, but some of them may wander a little, as shown in fig. 2, on the right.

During the spermatocyte division the neutral-red vacuoles come to lie opposite the chromosomes arranged on the spindle, as shown in Plate 15, fig. 4. The telophase is depicted in fig. 3, the group of vacuoles having been shepherded into two subequal groups, one of which goes to each daughter cell. In these cells, the Golgi dictyosomes are marked G, and undergo the changes which were worked out first by the writer, and subsequently by Bowen and Vishwa Nath. These bodies can be seen *intra vitam*.



TEXT-FIG. 5.—Spermatogenesis of *Saccorirrus*. A, full grown spermatocyte; B and C, division of secondary spermatocyte. Vacuome at Y. (From the 'Quart. Jour. Microsc. Sci.,' vol. 66 1922.)

In fig. 5 is the newly-formed spermatid, showing the mitochondria (M), Golgi bodies (G) and the vacuoles and centrosome. In fig. 6 the Golgi bodies have become stuck upon the nucleus preparatory to the secretion of the acrosome. The vacuoles nearly always hover nearby, but it has been impossible to show that they changed in staining ability, took part in acrosome formation, or were otherwise engaged in any activity at this period.



TEXT-FIG. 6.—Spermatogenesis of *Sacrowirrus*. A-E, spermatelocyst stages; vacuome at Y. (From the 'Quart. Jour. Microsc. Sci.,' vol. 66, 1922.)

In figs. 7 and 8, are two further stages in the formation of the spermatozoon, the neutral-red granules, exceptionally, being rather distant from the Golgi vesicle in fig. 8. By the later stage in fig. 9, the vacuoles and the Golgi bodies begin to drift down the spermatid, and ultimately many of the beads may be

found strewn along the tails of the ripening sperms, as in fig. 10. It was found impossible to say whether the vacuoles were completely stripped off the ripe sperms. In most arthropods the sperms are transferred to the female's *receptaculum seminis* in large bundles, with vacuoles and Golgi elements in the substance surrounding the bundles, and it is not easy to say whether either category passes into the egg with the sperm tail.

Helix Aspersa.*—This animal is very convenient for showing the vacuolar system. A very small piece of ovotestis is removed and placed in a drop of neutral-red Ringer. After a few minutes the piece is teased up with needles, and a cover-slip placed over it. The vacuoles "come up" in about 10 minutes as red intra-archoplasmic bodies, as shown in Plate 16, fig. 20. Such preparations will last many hours if ringed with vaseline. In fig. 21 is a later stage, corresponding to Mme. Karpova's figure on p. 308 of this paper, and on Plate 16, fig. 22, is the sperm residuum with the remains of the vacuolar system.

Cavia cobaya.—It has not been found possible to follow the neutral-red vacuoles from spermatocyte back into spermatogonia and germinal epithelial cells. In fig. 11 is a fully ripe spermatocyte, with neutral-red vacuoles near the archoplasm (AR) and Golgi cortex of the latter (G). In most spermatocytes the vacuoles lie near the Golgi apparatus in this manner. The archoplasm can often be stained pink, as indicated in fig. 11, so that the unstained Golgi cortex appears in profile when the whole sphere is focussed up and down under the microscope.

It is believed that these vacuoles occupied a position inside the sphere, as shown for *Helix*, in fig. 20, but during the spermatogonial divisions, or possibly while the early growth stages were in progress, the red vacuoles become extruded, as described for *Cerithium* by Monné (11). In the maturation divisions the same shepherding-out of the vacuoles into two groups occurs, as already mentioned in *Abraxas*. The metaphase and telophase stages are shown in figs. 12 and 13. In figs. 14–17 are the steps in the formation of the acrosome, from the material within the sphere.

At this period the neutral-red vacuoles keep near the region of the Golgi apparatus, fig. 15, being rather exceptional in this respect, and this case may be due to the manipulation of the cells of the testis during teasing. It is only at about stage fig. 18, that the vacuoles drift away from the Golgi apparatus.

The acrosome, from the time of its deposition on the nucleus, stains quite deeply in neutral-red, its two parts being differentiated clearly by stage fig. 16, though much earlier in fixed preparations (5).

* Many of the preparations I examined were made by my student, Miss M. O'Brien.

Jan Hirschler, in his most recent study in *Cavia*, figures rose or yellowish-red granules inside the sphere, from stages fig. 14 onwards to secretion of the acrosome. While it is certain that such granules are present (5) I have not been able to stain them up in the clear manner depicted by Hirschler and Monné.* Possibly their specimen of neutral-red was superior to those possessed by me, for I have tried a wide range of concentrations of neutral-red.

Discussion.

The first account of what is now recognised as a vacuolar system, together with the Golgi bodies and mitochondria, is that published by me early in 1922, in a description of the gametogenesis of *Saccocirrus* (4), from which text-figs. 6 and 7 are reproduced. At the time I was much puzzled by these bodies, and considered them to be some form of yolk granules. They stained greenish-brown in OsO_4 mixtures. Reference to text-fig. 6 shows that in the resting and dividing spermatocytes the granules Y form a group of discrete elements away from the other inclusions (G, Golgi elements; M, mitochondria). In the subsequent stages, in text-fig. 7, the vacuoles behave as described in this paper for other forms, and eventually pass into the tails of the spermatozoa, upon which they probably degenerate. Now chrome-osmium techniques only show the vacuolar system demonstratively under certain conditions, as in the large spermatid vacuome of *Notonecta* described by Voinov, and in the above-mentioned *Saccocirrus*.† The reason why the vacuolar system of this archi-annelid was noticed was because the vacuoles are filled with a denser material than is usual. They are, as I then claimed, of the nature of yolk spheres, and some of the yolk in the egg of *Saccocirrus* is undoubtedly of the same nature, as a re-examination of the slides concerned has shown.

There is little doubt that the vacuolar system, especially in insects, can be seen in chrome-osmium material, provided that the observer knows where to look for it. This is to say that the chrome-osmium techniques are quite adequate to fix the vacuoles, as vacuoles, but will not, it appears, allow of any sort of specific after-staining.† The virtue of neutral-red is that it stains the vacuolar system red, on a whitish or yellowish background of living protoplasm.

In his 1926 paper which elaborates the vacuome theory, Parat (13) mentions

* 'Zeit. f. Zellf. u. Mikr. Anat.,' 1928. This paper came into my hands after this present study was written.

† See footnote on p. 309.

that the intra-archoplasmic "vacuoles" of *Cavia* are to be considered as the "vacuome." At first the writer therefore tried to homologise the behaviour of the undoubted vacuolar system of *Helix*, which is intra-archoplasmic, with the *Cavia* spermatogenesis, but came to the conclusion that there were great difficulties in this task. This led to a personal investigation of the vacuolar system in *Cavia*, and it was easily shown that this mammal has a vacuolar system outside the archoplasm. Parat seems to have been homologising with the vacuome any intra-archoplasmic spaces or bodies, regardless of their staining properties.* The truth is that the vacuome is sometimes inside, sometimes outside, the archoplasm, and Parat never found the *Cavia* vacuolar system. It is just like that of *Saccocirrus* or of moths, and the intra-archoplasmic bodies of the cavy spermatocyte and spermatid are merely the pro-acrosomic secretions of the Golgi bodies, as Woodger and the writer claimed seven years ago.

The writer could find no evidence for believing that the vacuoles grew in size after separation from the Golgi bodies during spermatogenesis. There is nothing in the work of Mlle. Karpova, Monné, or Hirschler to show this, but in Voinov's case of *Notonecta* the granules do seem to grow in size—only this worker has not told us where the dictyosomes are during the growth of the spermatocyte. In any case the substance which grows in quantity inside the *Notonecta* vacuoles is not lipid, and the writer believes that the deposition of fatty materials within the vacuoles in oogenesis, and of zymogen during gland cell secretion, is brought about by the chromophil substance of the Golgi apparatus. Further careful studies in a form such as *Notonecta* should clear up these points. We remember Ludford's work in *Patella* oogenesis, in which he described the Golgi bodies as leaving the fatty vacuole after the latter had grown to its fullest size.

In 1924, Nassonow (12) showed that the protozoan contractile vacuole had a lipid cortex in all forms studied. He had endeavoured to show that this lipid cortex is the homologue of the lipid or chromophil part of the metazoon Golgi apparatus. Practically all modern cytologists are now disposed to believe Nassonow's views.

It seems almost inevitable that, after reading Nassonow's account of the lipid cortex or ring of the protozoan contractile vacuole, we must accept osmiophil membrane or palisade as the forerunner of the metazoon Golgi apparatus. We cannot forget that even in the metazoa the vacuole and cortex

* After prolonged immersion in stronger neutral-red Ringer, the pro-archoplasmic granules may go pink. The vacuolar system outside, however, goes deep red long before.

arrangement continually reappear. These facts bring us face to face with the evergreen problem of the function of the protozoan contractile vacuole.

Minchin ('Introduction to the Study of Protozoa') remarks: "As regards the function of the contractile vacuoles, it should be noted in the first place that their contents are always fluid and watery, and never contain solid particles of any kind. . . . The contractile vacuole is generally regarded as the organ of nitrogenous excretion, comparable functionally to the urinary organs of the Metazoa, but it is highly probable that the liquid discharged from it contains also the carbon dioxide produced by the respiratory process."

Minchin also mentions that contractile vacuoles are of common occurrence in free-living fresh-water Protozoa, usually wanting in marine forms and in entozoic or parasitic Protozoa. Degen, quoted by Minchin, states that the contractile vacuole has a membrane (a statement doubted by Minchin, but since proved correct by Nassonow) and that the function of the vacuole is to compensate for the tendency of the protoplasm to take up water by imbibition. In support of Degen's theory Zuelzer found that *Amæba verrucosa*, when transferred from fresh-water to sea-water, lost its contractile vacuoles. It would be interesting to repeat Zuelzer's work, with proper cytological investigations of the vacuole cortex. In the protozoa studied recently by the writer, the contractile vacuoles were not found to stain in a marked manner in neutral-red solutions.

Nassonow writes, "Der Exkretionsapparat der Protozoa verarbeitet in seinem Innern verschiedenartige Stoffe (sekretorische Funktion), welche aus dem Zellkörper ausgeschieden werden, wobei die lipoidofere Wand zur Isolierung dieser Stoffe während ihrer Bildung vom umgebenden Plasma dient."

In some of the diagrams in this paper it will be noted that the metazoon vacuoles are inside the archoplasm (*e.g.*, text-figs. 1 and 4), while in many others the system is outside. According to Nassonow's theory, the vacuolar system would perhaps be the homologue of the contractile vacuole of the ciliate or rhizopod protozoon, so that the archaic position of the vacuolar system may be assumed to have been inside the archoplasm, as in the mollusc cell drawn in text-fig. 4, G. In the Lepidoptera studied, in the younger spermatocytes, the vacuoles at first occupy the position just left by the dictyosomes* (Plate 15, fig. 1) and it seems that in many spermatocytes and spermatogonia it will be found that the vacuoles are first inside the archoplasm and later are deserted by the Golgi bodies. Nevertheless this supposed phylogenetic association between the two parts, vacuole and dictyosome, appears to explain why the vacuoles

* See 'Q.J.M.S.,' 1916.

keep near the dictyosomes during the latter's manœuvres at acrosome formation. There is probably some attraction between the two, for no one has been able to show that the wandering vacuoles take any part, directly or indirectly, in the formation of the sperm-head acrosome of any spermatozoa, except *Notonecta glauca*.

D. Voinov, in his paper on *Notonecta*, states: "Contrairement aux affirmations de Guilliermond, Parat et ses collaborateurs, il existe dans toutes générations de cellules sexuelles mâles de *Notonecta* et probablement dans toute autre cellule à côté et indépendamment du vacuome, un système golgien typique, représenté par des dictyosomes. Ils naissent dans les gonies, probablement au dépens d'une primordia"

It should be pointed out in justice to Parat, that this worker has recognised all three cell elements. His interpretations in this matter alone are faulty. Parat recognises both dictyosome and mitochondrion *intra vitam*, but he has blundered in attempting to homologise his vacuolar system with the argento-phil Golgi apparatus of Golgi's school.

It is not the purpose of this paper to enter into a lengthy discussion of our modern conceptions as to the structure of the cell. It may be said at once that in the spermatogenesis of all animals studied by the neutral-red techniques, a characteristic group of vacuoles has been shown to be present, even in such widely separated organisms as the common currant moth and the guinea-pig. In many somatic cells, too, we are obliged to admit the presence of a vacuolar system. Parat, to whom credit must be given for his insistence on the presence of such a system, has vitiated much that is good in his work by introducing the theory that such vacuoles are the Golgi apparatus, and by endeavouring to prove that the real Golgi apparatus is merely a group of modified mitochondria - his "lepidosomes."

It is for the cell physiologist to undertake experimental studies to ascertain the function of this recently recognised vacuolar system. If, as Bayliss* has written, neutral-red is a sensitive indicator for the presence of acid or alkali in the cell, it is a remarkable fact that the cell should contain a group of acidie vacuoles†, which in nearly all cases of spermatogenesis undergo a series of definite evolutions during growth, division and spermateleosis.

Neutral-red, like Janus-green, in weak solutions, is one of those dyes that is able to penetrate through living protoplasm, and stain objects included in it,

* 'Microtommists' Vademecum,' 1928.

† Why then do the vacuoles in fixed (Kolatchev) tissue still stain in neutral-red? It seems to me that the vacuoles contain some lipid material (September 30, 1928).

without seriously interfering with the processes of life. It does not usually stain the nucleus, until death supervenes, but in some cases, neutral-red does stain the living nucleus, *e.g.*, the heads of mammalian spermatozoa, which will move for hours in a stained condition in warm Ringer.

The sorting-out of the vacuolar system into two sub-equal groups at metaphase, as shown in this paper in Plates 15-16, figs. 1-21, is one of those vital phenomena about which we can explain nothing. There is no individual division of each vacuole, but by some means each of the four spermatids derived from the spermatocyte comes to have a group of vacuoles, in each case subequal in size. Presumably there is some form of protoplasmic streaming at the time referred to, but it is difficult to understand how such streaming could separate a group of vacuoles into two smaller groups of subequal size. Yet this undoubtedly occurs in all animals studied by Hirschler, Monné, and the writer, and the process can be watched happening *intra vitam*.

It is a curious fact that in such cases as the vacuolar system of *Saccocirrus* or *Notonecta*, for example, the individual vacuoles do not, after their separation from the dictyosomes, run together to form groups of larger vacuoles, or to make one large vacuole. That they may do so at a later stage when this is necessary, has been well shown by Voinov in *Notonecta* (text-fig. 3).

The work of Voinov demonstrates that in certain rare cases the extra-archoplasmic vacuolar system plays an important rôle in spermatogenesis, yet the majority of the descriptions of this cell structure indicate plainly that the "vacuome" is passive in nature, and it is probably only in oogenesis and gland secretion that it serves in an important capacity as a mechanism for yolk and zymogen secretion.

A point which must be studied is how the vacuoles increase in number. The work of Hirschler, Ludford, Miss King, Brambell, Nath, Bhattacharya, and the writer, has demonstrated clearly that the fatty yolk is usually associated with the Golgi bodies, and possibly in such cases, each Golgi element, when dividing, gives a part of the vacuole to the daughter Golgi elements. It has not been possible to show that the Golgi element is unable to produce a vacuole of its own, and the writer has constantly suggested that the dictyosome has the power of forming a vacuole for itself.

The work of Miss A. G. Hill in this laboratory has convinced the writer that in such examples of oogenesis as that of *Daphnia*, the Golgi element is a cortex on the vacuole, and the division of the element brings about a division of the associated vacuole.

It is necessary to come to some conclusion as to the exact status of the vacuolar

system. Some authors write of the vacuome as if it were a third cell constituent, of the status of the mitochondria. The writer and his pupils have constantly recognised, in eggs especially, vacuoles filled with fat or yolk, but supposed to have been produced by the chromophil part of the Golgi apparatus. There is nothing in recent work in the vacuolar system which shakes the writer's belief in the correctness of this view. The vacuolar system is produced by the Golgi apparatus, but may, as in the case of the male cells, be early separated from the dictyosomes. There is no evidence that the vacuolar system can, *per se*, increase afterwards the number of its vacuoles.

All the evidence of oogenesis and gland secretion points to the conclusion that the chromophil parts of the Golgi elements are able to produce new vacuoles, and to fill them with fat and zymogen. In many cases as has been indicated above, the number of vacuoles corresponds to the number of dictyosomes. There is nothing in the work of Nassonow or other writers which leads us to believe that the contractile vacuole is ever completely separated from the chromophil cortex. Even in the case of the peculiar halo-shaped Golgi system of *Dogielella* (12), the latter keeps in close association with the vacuole.

It seems to be only in the male cells, where in almost all cases no direct connection between extra-archoplasmic vacuome and acrosome can be traced, that the vacuolar system is dissociated from the dictyosomes at an early period. The writer therefore considers that the vacuolar system is produced by the Golgi apparatus and is of a lower status than the latter. The vacuoles seemingly do not increase in number without the intervention of the Golgi cortex, nor is there yet any really satisfactory evidence that zymogen or yolk can accumulate in them without the assistance of the Golgi system.

General Summary.

1. There exists in the animal cell a vacuole or system of vacuoles primitively associated with, and probably produced by, the argentophil cortex of the Golgi apparatus.

2. Recently Hirschler, Monné, Voinov and others have shown that this system of vacuoles may be separate from the Golgi apparatus during spermatogenesis. [Examples described by the writer are the Lepidoptera (*Abraxas*, etc.), Mollusca (*Helix aspersa*), and Mammalia (*Cavia cobaya*).]

3. This fact was first demonstrated by the writer in *Saccocirrus* in 1921, though the nature of the bodies in question was not then properly understood.

4. Recent work has all gone to show the unsoundness of Parat's vacuome theory. The vacuole is not the Golgi apparatus, but the associate or derivative of the Golgi cortex, and the dictyosome is not a modified mitochondrion as suggested by Parat, but is a separate and characteristic structure.

5. The vacuolar system may be situated inside, or outside the archoplasm. In mollusca it is usually inside the archoplasm. In such a case at mitosis it usually forms a scattered group of vacuoles near each aster.

6. In the case of the vacuolar system which lies outside the archoplasm, the vacuoles form a spindle-shaped group near the chromosomes, but outside the area of the amphiaster. This group comes to be divided into two smaller groups, which keep near the centrosomes at telophase, and so in the daughter cells each group lies near the re-formed Golgi apparatus.

7. At spermatogenesis in the type of cell with a spindle-shaped group of vacuoles outside the archoplasm, the latter keeps near the Golgi apparatus, and follows it during its evolutions in the formation of the acrosome. No evidence has been found to indicate that the extra-archoplasmic vacuoles take any part in the formation of the acrosome, except by Voinov in *Notonecta glauca*, which seems to be unique.

8. It appears to be during oogenesis and gland-cell secretion that the vacuolar system functions most importantly. It is then closely related to the chromophil substance of the Golgi apparatus.

9. It is not believed by the writer that the vacuolar system is of the status of a third cytoplasmic inclusion separate from the Golgi bodies. During the spermatogenesis of some animals (*e.g.*, *Saccocirrus*, *Cavia* and *Abraxas*) the vacuoles are discarded early by the mother Golgi bodies, and hover apart during the spermateleosis stages.

10. In oogenesis the Golgi elements are supposed by the writer to be able to produce vacuoles and to condense in them such substances as fats and lipins, and zymogen (in glands).

11. In the case of *Notonecta*, as described by D. Voinov, the spermatocyte vacuome is diffuse, and the non-lipoid acidic substance in the vacuoles appears to increase without direct contact with the dictyosomes, though this point has not been cleared up; it has not been shown that lipoid, found in the vacuome of eggs, can be produced without contact with the argentophil part of the dictyosome.

12. It has been stated by Parat that in *Cavia* the pro-acrosomic spaces, or granules, are the vacuome. It has, however, been shown in this paper that the pro-acrosomic granules do not readily stain deeply in neutral-red, but that a

vacuolar system exists outside the archoplasm, resembling exactly the type found in *Saccocirrus* and *Lepidoptera*.

13. Attention is drawn to the fact that Bowen has described a new cell constituent in plants. Part of Bowen's work has been gone over by the writer's students, and the suggestion made by Bowen that this new cell constituent represents the Golgi apparatus is supported by the writer's observations.

14. The view of Guilliermond, Mangenot, Dangeard, Parat and other French writers, that the vacuolar system in plants is the homologue of the metazoon nerve-cell Golgi net, is not upheld by recent researches.

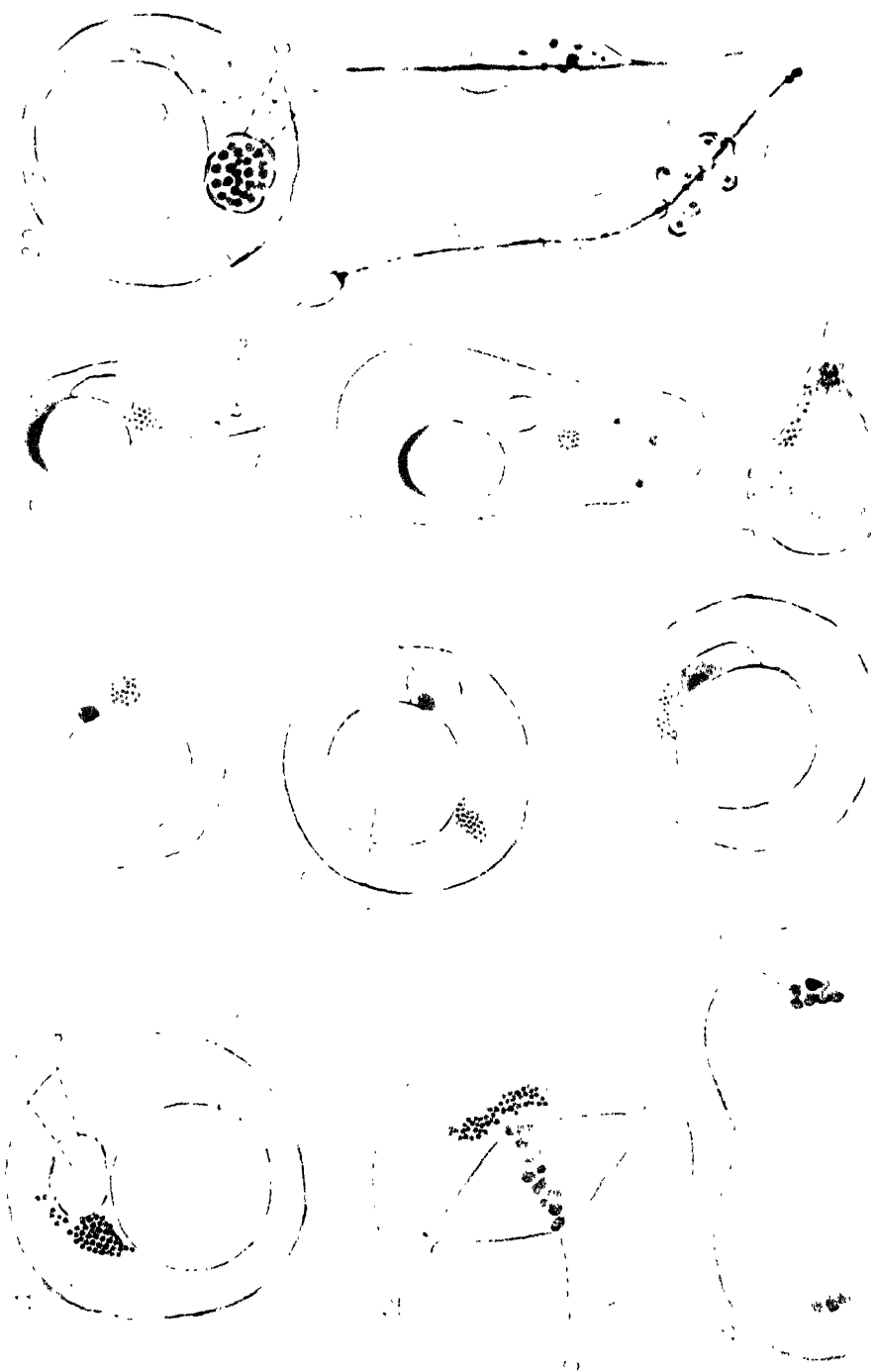
15. The plant cell appears to be organised on the lines of the animal cell, with Golgi dictyosomes (platelets), mitochondria, and perhaps a vacuolar system, but, in addition, a plastid system. A great deal more work on plants is necessary.

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* Very full bibliographies of the literature on the Golgi apparatus, etc., appear in the recent American text-book edited by E. V. Cowdry, and entitled "Special Cytology."





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DESCRIPTION OF PLATES.

Lettering.—A, acrosome. AR, archoplasm. C, centrosome. CH, chromosome. EG, Von Ebner's granules. F, filament. G, Golgi apparatus. M, mitochondria. V, vacuolar system.

PLATE 15.—*Abraxus Grossulariata*.

FIGS. 1 and 2.—Spermatocytes.

FIGS. 3 and 4.—Maturation divisions.

FIGS. 5 to 10.—Spermateleosis.

PLATE 16.—*Uria Corbaya*, 11-19. *Helix Aspersa*, 20-22.

FIG. 11.—Spermatocyte.

FIGS. 12 and 13.—Maturation divisions.

FIG. 14 to 19.—Spermateleosis.

FIG. 20.—*Helix* spermatocyte.

FIGS. 21 and 22.—Spermateleosis.

The Effect of Glare on the Brightness Difference Threshold.

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(Communicated by Sir John Parsons, F.R.S.—Received January 1, 1929.)

[From the National Physical Laboratory.]

Introduction.

By the brightness difference threshold is ordinarily understood the least perceptible difference of brightness of two contiguous test-fields when the brightness of one of these has a given value. It is well known, however, that this critical brightness difference depends not only on the given test-field brightness but also on the brightnesses in the remaining parts of the visual field. A change in the latter is accompanied in general by a change in the threshold.* When an unshielded light source is introduced into the field of view, the field brightness being otherwise unchanged, the threshold is found experimentally to increase. The observed increase in this case may be regarded as one of the things intended when the unshielded light source is described as “glaring.” The researches of Bordoni and others† have shown that the change in the threshold varies continuously with the intensity of the glare source and with its position in the field of view. The important conclusion has also been arrived at that provided the linear dimensions of the glare source do not exceed a certain limit, the effect on the threshold depends only on the candle-power in the direction of the subject’s eyes and not explicitly on the size or brightness of the source. When such is the case, we may speak of a point source of glare and specify it merely by its position in the visual field and its candle-power.

In the present paper an account is given of the determination of the threshold in the presence of a point source of glare for various values of the general brightness level in the field, and for different positions and intensities of the glare source. The ranges of the variables have been chosen with a view to the application of the results in practical illumination problems, such as the design of street lighting installations to give best visibility conditions. The method of measurement is chosen to correspond as far as possible to normal vision conditions, the subject having the unrestricted use of both eyes. In a number of the earlier threshold determinations, notably those of König and Brodhun,

* The “brightness difference threshold” will be referred to throughout the remainder of this paper simply as the “threshold.”

† Bordoni, ‘*Elettrotecnica*,’ vol. 11, p. 585 (1924); Holladay, ‘*Journ. Op. Soc. America*,’ vol. 12, p. 271 (1926), vol. 14, p. 1 (1927).

the chief interest was the retinal sensitivity, and pupillary accommodation and binocular effects were eliminated by using one eye only and an artificial pupil. The threshold appropriate to these conditions will not necessarily be the same as that for normal vision conditions.

The Observational Conditions.

We commence with a description of the conditions of measurement from the subject's point of view. This amounts virtually to a definition of what is being measured.

The subject is seated and looks through a rectangular aperture (7 inches by $2\frac{1}{2}$ inches) into an otherwise totally enclosed cabinet, the sides and roof of which are formed by black curtains. Directly opposite the subject's eyes and at a distance of 4 feet therefrom, a square aperture in the curtaining of 2 feet 6 inches side, discloses a whitened screen. Under "absence of glare" conditions, the square field so formed is uniformly bright, except that in the centre a circular test spot of 1.7 inch diameter has a uniform brightness either greater or less than that of the surrounding field. The difference of brightness between central test spot and surrounding field is continuously variable by the experimenter independently of the subject. Under glare conditions, the square field includes, in addition to the central test spot, a circular "glare spot," 0.39 inch in diameter, which may be situated at any part of the field. The brightness of the glare spot is variable, and is in general much greater than that of the main field.

A photograph of the field as seen by the subject is reproduced in fig. 1.

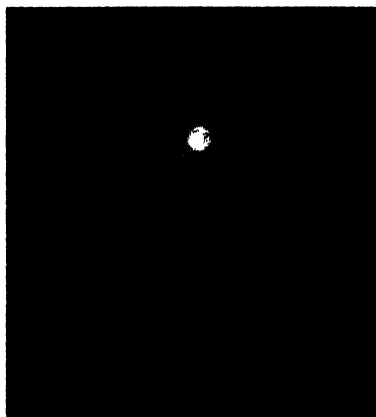


FIG. 1.—Photograph of the experimental field as seen by the subject. Only the central region is shown, including the glare and test spots.

For a given field brightness, and for a given position and intensity of the glare spot, the difference of brightness between the central spot and the surrounding field can be so reduced that the test spot is absolutely invisible to the subject. It is required of the subject :—

- (1) As the difference of brightness steadily increases from zero, to signal when the test spot becomes just visible.
- (2) As the difference of brightness steadily decreases from an easily visible value, to signal when the test spot just ceases to be visible.

A polar diagram of the subject's visual field (binocular) under the experimental conditions is shown in fig. 2. The dotted line represents the normal

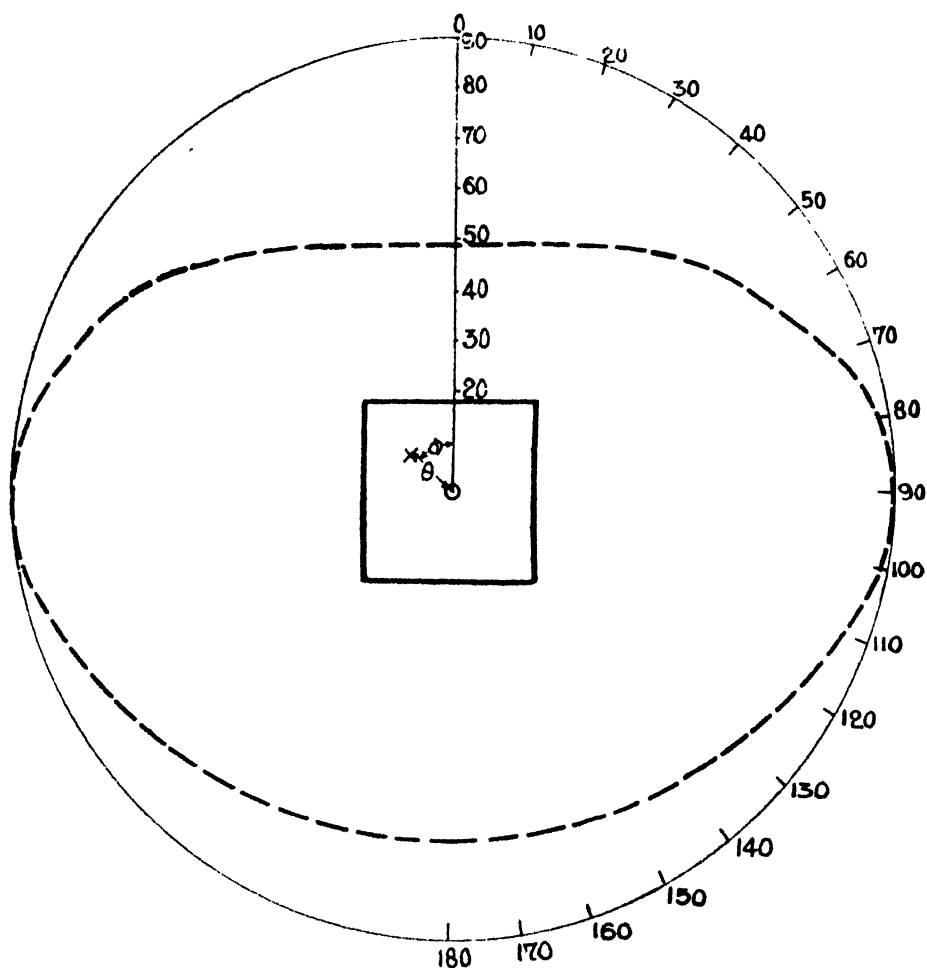


FIG. 2.

limit of vision. At the pole, or fixation point, is the test spot subtending an angle of 2° at the eyes. Concentric with the pole is the square "background field," the size of which is most simply expressed in terms of the diameter of the circle having the same area. In our case this subtends an angle 2θ of approximately $40'$ at the eyes. The position of the glare spot is determined by the polar angle θ and the azimuthal angle ϕ . The glare spot is circular and its diameter subtends an angle $\delta_G = 0.47^\circ$ at the eyes. The brightnesses of the different parts of the field are denoted as follows :

B_0 = brightness outside the background field.

B_s = brightness of background field.

B_c = brightness of central test spot.

B_G = brightness of glare spot.

When B_c is greater than B_s corresponding to any set of values of B_0 , B_s , B_G , θ and ϕ two critical values of $B_c - B_s$ are determinable for which the test spot is (a) just visible to the subject as $B_c - B_s$ is increased from zero ; (b) just invisible to the subject as $B_c - B_s$ is decreased from an easily visible value. The critical values of $B_c - B_s$ for these two cases will be written T_{ba} and T_{bd} respectively, the suffixes denoting that the test-spot is brighter than the background field and that the critical values are for appearance and disappearance of the test-spot. When $B_c < B_s$ it is not always the case that a similar pair of thresholds is determinable, for under sufficiently intense glare conditions the difference of brightness between centre spot and the given background brightness may be inadequate to render the test-spot visible even with $B_c = 0$. In the absence of glare, and for the less intense glare conditions, T_{da} and T_{dd} are defined as the critical values of $B_s - B_c$ for $B_s > B_c$ and for appearance and disappearance respectively.

For some purposes it is desirable to work with the threshold expressed as a percentage of the background field brightness, a quantity corresponding to the Fechner Fraction of earlier investigations. We put therefore $F_{ba} = T_{ba} 100/B_s$ and define F_{bd} , F_{da} , F_{dd} similarly.

Another method of presenting the results will also be used in which the mean threshold of appearance and disappearance $T_b = \frac{1}{2} (T_{ba} + T_{bd})$ and the proportional separation of the thresholds $k_b = \frac{(T_{ba} - T_{bd})}{\frac{1}{2} (T_{ba} + T_{bd})}$ are employed.

It is convenient to express the intensity of the glare spot in terms of the illumination incident on the plane occupied normally by the pupils of the subject's eyes. This illumination E_G is given in terms of θ , B_G , the area of the

glare spot a_G and the distance d of the subject's eyes from the screen (in our case 4 feet), by the formula

$$E_G = \frac{a_G}{d^2} \cos^4 \theta B_G.$$

The following five magnitudes provide an adequate specification of the field conditions corresponding to a particular threshold determination: B_0 , B_s , θ , ϕ , E_G . All the results discussed, however, refer to conditions in which $B_0 = 0$ and $\phi = 0$.* It will be necessary, therefore, to give only B_s , θ and E_G .

Description of Apparatus and Method.

The experimental arrangement is shown diagrammatically in figs. 3 and 4. The lamps L_1 and L_2 , enclosed in whitened cubes B_1 and B_2 , illuminate the square of the whitened screen S , exposed by the aperture in the curtains forming the cabinet C . Filters can be placed in front of the apertures of the cubes B_1 and B_2 to give various illuminations of the screen S .

In addition to the approximately uniform "background" illumination produced in this way, the screen also receives the "difference" illumination which is projected on to it by a suitably devised optical arrangement.

The lamp l is mounted on a carriage which runs in grooves on an extended bench so that the distance of the lamp from the conjugate plane F of the screen S with respect to the lens P can be varied continuously. In the plane F is mounted a glass slide divided into two parts; one part is opaque except for a small circular area in the centre which is clear, and the other portion is clear except for a small opaque disc of the same diameter as the clear area of the first part. The slide moves in grooves so that either the clear disc or the opaque disc can be brought on to the axis of the projector lens P . In the former case the lens throws on the screen S a circular bright spot and in the latter the entire field is illuminated except for a circular area in the middle which is dark. In both cases the difference of brightness between the central spot and the surrounding field is proportional to the luminous flux in the plane F due to the lamp l .

The glare spot is produced by a hole H in the screen behind which a lamp G is set up. A piece of ground glass is mounted in the hole H and the position of G is adjusted so that the blurred image of the filament of the lamp G formed by the hole H falls on the viewing aperture A . A Holophane lumeter U_1 serves to measure the brightness of the screen S at the centre of the square

* Glare spot vertically above test spot.

field. A similar lumeter U_2 is sighted on the viewing aperture and is used to measure the illumination of the eye due to the glare spot. For this purpose a strip of previously calibrated opal glass is slid over the aperture.

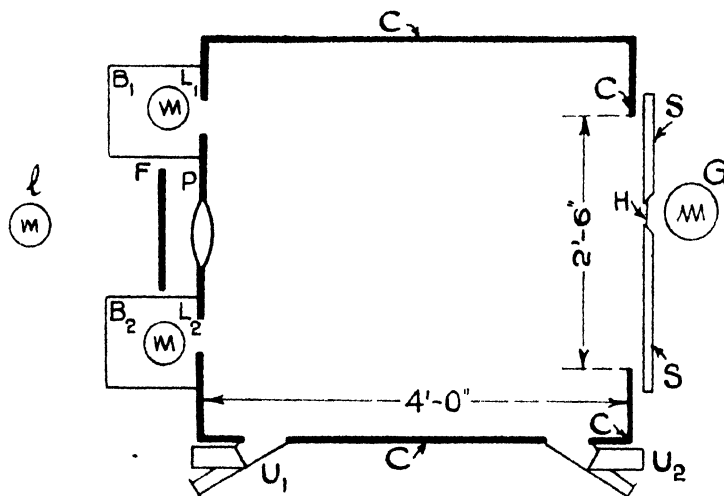


FIG. 3.

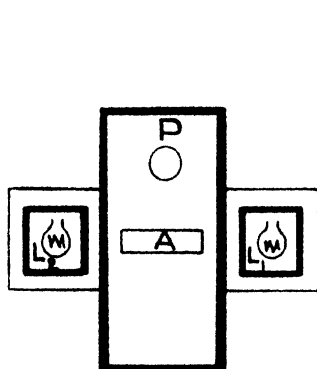


FIG. 4.

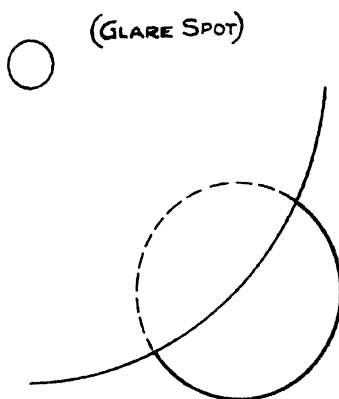


FIG. 5.

(TEST SPOT.)

The subject sits with his head between the cubes B_1 , B_2 and underneath the bench carrying the lens P and the lamp l . His eyes are effectively screened from any light other than that passing through the aperture A . In making a determination of the critical brightness difference of appearance, the lamp l is drawn in slowly until the subject signals that he can see the spot. The lamp

is then drawn in so that the spot is easily visible and finally gradually withdrawn for the disappearance observation.

All the lamps mentioned in the above description are gas-filled lamps run at their rated voltage (100 volts). Suitable electrical arrangements enable the voltage on each lamp to be checked and adjusted periodically during the course of a series of measurements. Using 500-watt lamps L_1 , L_2 a brightness of the screen equal to 20 candles per square foot is obtainable. Most of the background brightnesses worked with are considerably lower than this and 30-watt fullolite lamps and filters are used. For the glare illumination at the eye, a value of about 1.0 foot-candle is obtainable with a 500-watt gas-filled lamp for G. The difference field lamp l has to be chosen for each background brightness and glare condition, to give a sufficient range of variation on either side of the critical settings. The difference field brightness produced on the screen is determined for one position of the lamp and the inverse square law is applied to calculate the brightness at any other position, the validity of this procedure having been established experimentally. When measuring the difference field brightness the background lamps are of course switched off.

Notes on the Method.

The special features of the method may be summed up as follows :—

- (1) The subject uses both eyes and has an unobstructed view of the field over a wide angle. He is not required to make a setting, but only to give a judgment.
- (2) The optical projection method of producing the test object ensures that the outline of the test-spot exists for the observer only in as much as he can detect the difference of brightness between the two fields. When the difference of brightness falls below the threshold value, there is no "line" to show where the two fields meet.

The notions "just visible" and "just invisible" which underlie the measurements are not, of course, sharply defined. It should be noted that the subject is called upon to say when *the spot* becomes just visible or just invisible. Preliminary work in the absence of glare had shown that not infrequently the subject would have an idea that he could see something not recognisable as a circular spot, at brightness differences very much lower than were normally obtained. Although experiment appears to confirm that before the spot as such becomes visible, there is a suggestion of unevenness in the brightness of the centre of the field, yet this vague indication scarcely constitutes a workable

criterion, for at times the subject imagines he can detect something when the difference field has been, unknown to him, completely cut off. It is stipulated, therefore, that the spot itself shall be just visible or just invisible as a circular form.

Under glare conditions the matter is complicated by the fact that the parts of the test-spot are at different distances from the glaring source, and though the more distant parts may be clearly seen yet those nearest the glare source remain invisible. It was suggested to subjects that they should consider the spot as just visible when they could just discern the outline of the half of the spot furthestmost from the glare spot (see fig. 5). It may be imagined this is not an easy criterion to apply and we shall expect, under glare conditions, the precision of the measurements to suffer.

Of the four threshold magnitudes T_{ba} , T_{bd} , T_{da} , T_{dd} the first two must be regarded as measuring a simpler characteristic of a given set of glare and background conditions. As already mentioned, for intense glare conditions T_{da} and T_{dd} do not exist. Furthermore, in determining T_{da} and T_{dd} the difference of brightness between spot and surround is changed by varying the background field brightness B_s . In the absence of glare the range of variation is small compared with the absolute value of the background brightness, but in the presence of intense glare this is no longer the case and changes in the state of adaptation of the retina will accompany the variations in the test-spot brightness difference. The conditions for the T_{da} and T_{dd} determinations are therefore more complex than those for the thresholds T_{ba} and T_{bd} . For this reason the great majority of the measurements have been made for the latter quantities. It is unlikely that any essentially new features of the problem will be disclosed from a consideration of the more complex behaviour of T_{da} and T_{dd} .

There is one factor which is left uncontrolled in making the determinations and which may have an effect on the results, namely, the speed with which the difference of brightness between the test-spot and the surrounding field is varied. To arrange for this difference of brightness to vary at a known constant rate would have involved extra complication of the apparatus which it was considered desirable to avoid, at least in a first investigation.

The present procedure using a continuously varying brightness difference was not adopted without an investigation of a "discontinuous" and at first sight perhaps more attractive method. In the discontinuous method the subject is confronted with the background brightness only. A specially designed shutter then cuts off the subject's view for a very brief interval (less

than $1/5$ second) and during this period the difference brightness is superimposed on the background brightness. The subject is given 5 seconds in which to decide if he can see the spot, after which the shutter is dropped again and the background brightness alone is exposed. By making a number of exposures at various difference brightnesses the position of the threshold may be determined. The results of measurements on these lines were contradictory and difficult to interpret. One general conclusion may perhaps be drawn. At the initial instant when the subject is confronted with the combined field, he is able to detect brightness differences which, after a few seconds, are no longer visible to him.* The continuous method has the advantage that it excludes the possibility of any such "instantaneous effect," and chiefly for this reason was adopted.

The Subjects.

The following details concerning the subjects are submitted as having a possible bearing on the results obtained. All are members of the staff of the Photometry Department of the National Physical Laboratory, and the following table indicates the photometric experience of each.

Table I.

Subject.	A. (B.J.O.)	B. (G.C.C.)	C. (G.E.V.L.)	D. (C.J.M.)
Experience of general photometric work.	6 years	8 years	6 months	2 years
Experience of brightness threshold determinations before the observations submitted in this paper.	6 months	6 months (intermittent)	Nil	Nil

In addition, subject A took part in all preliminary work using continuous and discontinuous methods and may be said to have had the most experience of this type of observation.

Frequency Curves for the Threshold.

A preliminary investigation was made of the "precision" with which the threshold is determinate. For this purpose a number of series (in each series 20 sittings, $5F_{ba}$ and $5F_{bd}$ readings at each sitting) for given conditions and subject were taken and frequency curves constructed for each series showing

* This effect may be connected with the phenomenon of overshoot of visual sensations investigated by Broca and Sulzer and others. See Walsh's "Photometry," p. 60 (1926).

the distribution of the results. To obtain reasonably smooth frequency curves the fundamental interval chosen equalled 10 per cent. of the mean value of the 100 readings of the set. The number χ of actual observations in each such interval was determined and plotted against the value of the threshold corresponding to the mid-point of the appropriate interval.

Frequency curves were obtained for the four subjects in the absence of glare and with the following values of the background field brightness :—

High brightness level	$B_s = 0.26$ or 0.55 .	} candles per square foot.
Intermediate brightness level	$B_s = 0.030$.	
Low brightness level	$B_s = 0.0037$	

The curves for the intermediate brightness level are shown in fig. 6 and are a fair sample of the results obtained. About half of the 24 curves obtained show a single peak, are not grossly asymmetric, and approximate to normal frequency curves. Of the remainder, 3 or 4 show double peaks. In the case of the curves showing an approximately normal form, the abscissa of the maximum ordinate may fairly be taken as the value of F_{ba} or F_{bd} corresponding to the set of readings, and the half-band-width (the width of the curve at a height equal to half the maximum ordinate), as a measure of the precision with which F_{ba} or F_{bd} is determinate. For the other curves, and particularly for those showing double peaks, these notions fail. Two interpretations of the double peak form suggest themselves: (i) the threshold may be constant but complex, *i.e.*, there may be two thresholds, the observer using one or other for each observation, the relative probabilities corresponding to the heights of the two peaks; and (ii) the threshold although simple may have altered during the course of the set of readings.

A further statistical analysis* of the observations suggests that the latter is the more likely explanation and that view will be adopted here. The variations in the threshold observations are to be regarded therefore as made up of (a) random variations about an "instantaneous" threshold value, the spread corresponding to the half-band-width of the approximately normal curves; (b) shift of the "instantaneous" value during the course of the observations. Probably if enough observations were taken over an extended period the shifting of the instantaneous threshold would also be equivalent to a random variation and again a normal single peak frequency curve would result, although of half-band-width greater than that for the instantaneous value.

* For advice and comments on the statistical aspects of the investigation, the author's thanks are due to Miss E. M. Newbold.

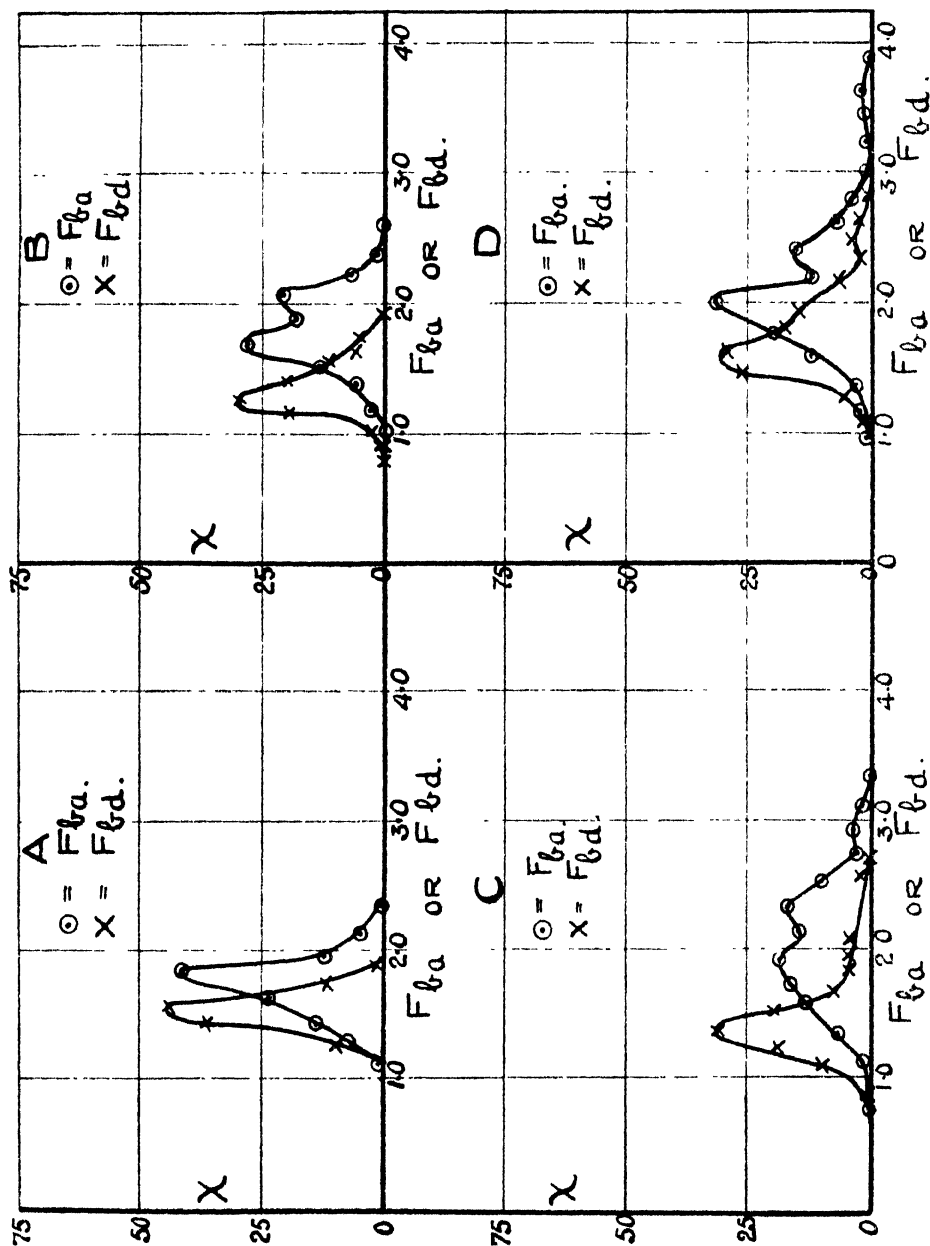


FIG. 6.

For the average value of the precision ratio (half-band-width)/(abscissa of maximum ordinate) for the approximately normal frequency curves the figure 0.25 is obtained. The half-band-width of the normal frequency curve $y = e^{-h^2(x-a)^2}$ is given by $\frac{2}{h} \{\log_e 2\}^{\frac{1}{2}}$ and the precision ratio by $\rho = \frac{2}{ah} \{\log_e 2\}^{\frac{1}{2}}$.

The probable error for the normal curve equals $\frac{0.477}{h}$ or expressed as a percentage of the mean $\frac{0.477}{h} \frac{100}{a}$. Thus a precision ratio ρ corresponds to a

percentage probable error $\rho \frac{47.7}{2 \{\log_e 2\}^{\frac{1}{2}}} = 28.6 \rho$. For $\rho = 0.25$, the probable

error of a single observation is therefore 7.2 per cent. The uncontrolled fluctuations in the physical conditions (such as slight changes in candle-power of lamps due to voltage variation, etc.) may be assumed to be such that the resulting deviations in the value of F_{ba} or F_{ab} which are greater than 5 per cent. do not exceed 10 per cent. of all the deviations from this cause.

It is readily shown that such fluctuation corresponds to a probable error of 2 per cent. If z is the probable error associated with the subjective indefiniteness of the instantaneous threshold, we have by the law for compounding probable errors (assumed independent) $7.2^2 = z^2 + 2.0^2$, which gives $z = 6.9$ per cent.

No definite estimate of the range of the shift variations in the instantaneous threshold can be derived from the data available, although it appears in some cases to be as much as 25 or 30 per cent. For the best representative value of the threshold in the general case, the arithmetic mean is most suitable. This is the same as the abscissa of the maximum ordinate for the approximately normal frequency curves.

It will be noticed in fig. 6 (and the same is true for the other frequency curves not reproduced here) that for a given background field brightness and a particular subject the curve corresponding to the appearance threshold is displaced to the right with respect to the curve corresponding to the disappearance threshold. This result is to be expected. The subject having once seen the spot is still conscious of seeing it when the brightness difference is reduced to a value at which he is unable to pick it up as the brightness difference is increased from zero.

Frequency curves for subjects A and D, obtained from sets of observations under glare conditions, are shown in fig. 7. Concurrent absence of glare curves with the same background field brightness were obtained by taking alternate sittings with and without glare. The glare spot for these measurements was situated immediately above the test-spot and at a distance between centres of about 2 inches ($\theta = 2.5^\circ$), the illumination produced at the pupillary plane being about 0.2 foot candles and the background field brightness about 0.03 candles per square foot. The observations extended over three days.

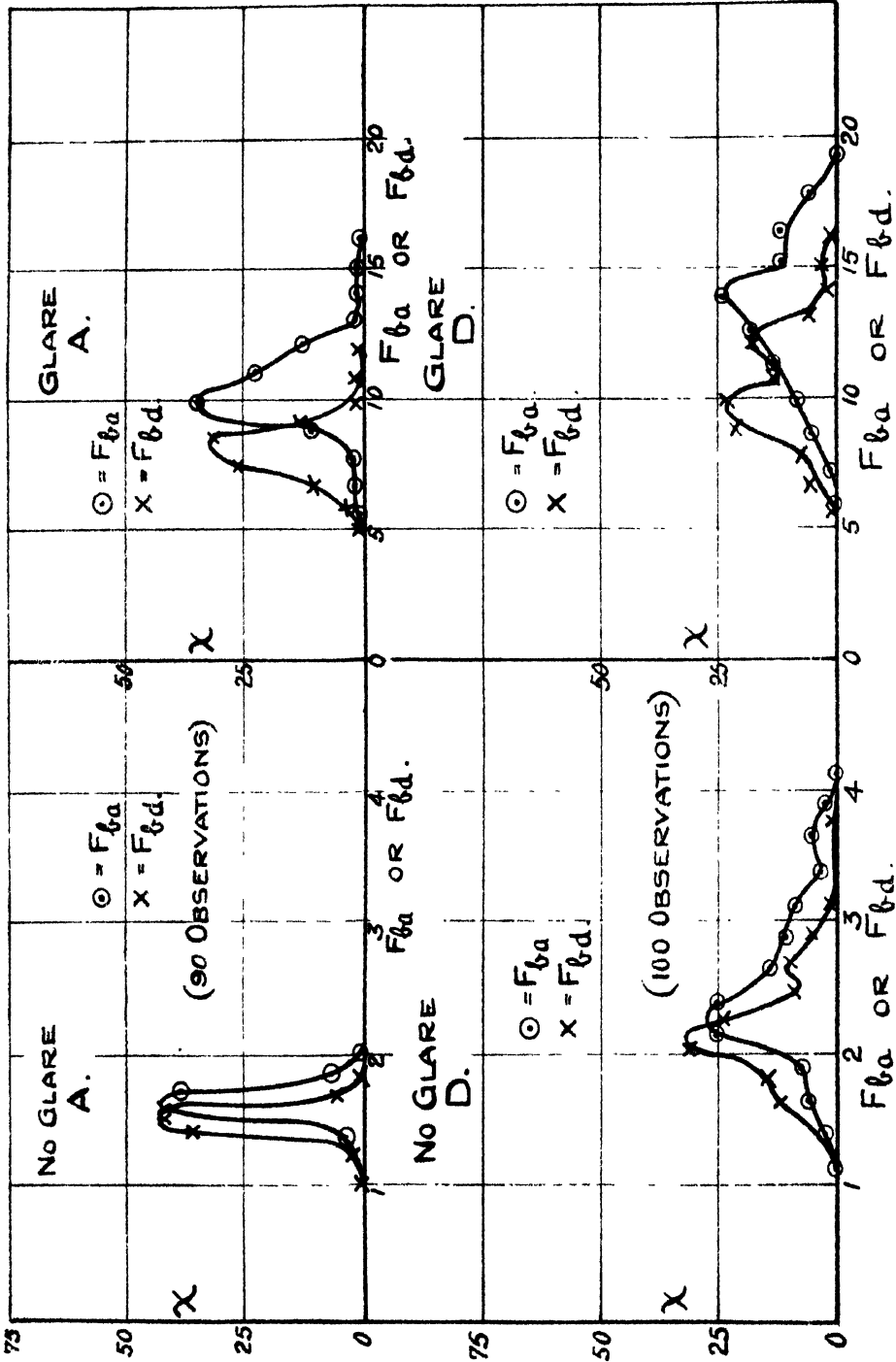


FIG. 7.

The general remarks already made about the frequency curves in the absence of glare, also hold good for those obtained under glare conditions, except that the proportion of normal curves is less. The percentage separation between appearance and disappearance thresholds appears in most cases to be greater in the presence of glare.

It is remarkable that for subject A the frequency curves are nearly all of the approximately normal shape, whereas for subject C shift of the instantaneous threshold during a series of measurements usually occurs. These subjects are respectively the most and least experienced photometric observers. The results for subjects B and D support the idea of a relation between threshold shift and inexperience. It is plausible to suppose that the inexperienced subject finds difficulty in acting as a passive receptor of his visual impressions. Any straining to see the test spot or conscious doubt of the evidence of his vision, will probably result in the establishment of a criterion very susceptible to changes in the general psychological condition of the subject. Even with the most experienced subject it is to be expected that variations will occur from possible physiological changes in the eyes, but it is probable that these would be more easily traceable to such causes as fatigue, ill-health, etc.

Before passing to the main results it may be pointed out that frequency curves for F_{da} and F_{dl} in the absence of glare showed the same characteristics as those for F_{ba} and F_{bl} . In addition for a given B_s the value of the F_{ba} and F_{bd} thresholds as measured by their mean equalled approximately that for the F_{da} and F_{dl} thresholds.

The Main Measurements.

The measurements were restricted to determinations of T_{ba} and T_{bd} for the subjects A and B, the ranges of the variables being as follows :—

B_s : 0 to 1 candle per square foot. E_g : 0 to 1 foot candle.

θ : 1° to 10° .

The observations were obtained in day runs. In such a run 10 or 11 sittings, corresponding to different conditions, were obtained for each subject. Six pairs of observations of T_{ba} and T_{bd} were made at a sitting, the first pair being rejected to eliminate anomalous results obtained before the subject became properly adapted. In the no-glare runs a different background brightness B_s for each sitting was employed. For a run in the presence of glare, the variable condition was the glare illumination at the eye, background brightness and glare angle being kept constant. The glare runs were in every case com-

menced and terminated by sittings with zero glare. The arithmetic mean of the five readings of T_{ba} or of T_{bd} obtained at a sitting will be referred to as a 5-mean.

No-glare Results.

The mean results of five runs in the absence of glare are summarised in Table II. Eleven different background brightnesses were worked with, and the five means for each brightness, obtained in the different runs, have been averaged to give the values shown. The curves of fig. 8 show the variation of the Fechner Fraction $F_b = \frac{F_{ba} + F_{bd}}{2}$ with $\log_{10} B_s$. Attention may be drawn to two interesting features of these curves.

Table II.

		Subject A.				Subject B.			
B_s	$\log_{10} B_s$	T_{ba}	T_{bd}	T_b	F_b	T_{ba}	T_{bd}	T_b	F_b
		$\times 10^{-3}$	$\times 10^{-3}$	$\times 10^{-3}$		$> 10^{-3}$	$\times 10^{-3}$	$\times 10^{-3}$	
0.00104	3.017	0.080	0.051	0.066	6.4	0.099	0.058	0.078	7.5
0.0052	3.716	0.164	0.107	0.136	2.63	0.169	0.107	0.138	2.75
0.0105	2.021	0.264	0.157	0.210	2.00	0.276	0.160	0.218	2.08
0.0207	2.316	0.394	0.259	0.326	1.58	0.428	0.259	0.344	1.66
0.0424	2.627	0.739	0.476	0.608	1.43	0.780	0.461	0.620	1.46
0.0952	2.979	1.13	0.763	1.00	1.05	1.43	0.789	1.11	1.17
0.124	1.093	1.57	1.04	1.30	1.05	1.87	1.08	1.48	1.19
0.265	1.423	2.73	1.82	2.28	0.86	3.00	1.84	2.42	0.99
0.520	1.716	5.50	3.29	4.40	0.85	5.51	3.10	4.30	0.83
1.08	0.033	8.92	4.82	6.87	0.64	8.60	4.57	6.58	0.61
2.16	0.334	28.6	16.6	22.6	1.05	28.4	15.6	22.0	1.02

N.B.—For convenience, the numbers in the T columns give, not candles per square foot but millicandles per square foot. This practice is adhered to throughout. The 10^{-3} is, however, always shown at the top of the column, as the unit in which T is expressed is formally candles per square foot.

In the first place, at $B_s = 1$ candle per square foot for both subjects the Fechner Fraction is as low as 0.60 per cent. This means that using both eyes normally both these subjects could detect a percentage difference of brightness of about a third the minimum percentage difference 1.7, detected by König in his classic experiments. This result is also true for the writer and for two other subjects. The second point of interest, is the well-defined upward turn of the curves, when a background brightness of 1 candle per square foot is exceeded. The increase in Fechner's fraction is accompanied by the setting in of an unpleasant dazzling sensation, similar to that of snow on a very bright day.

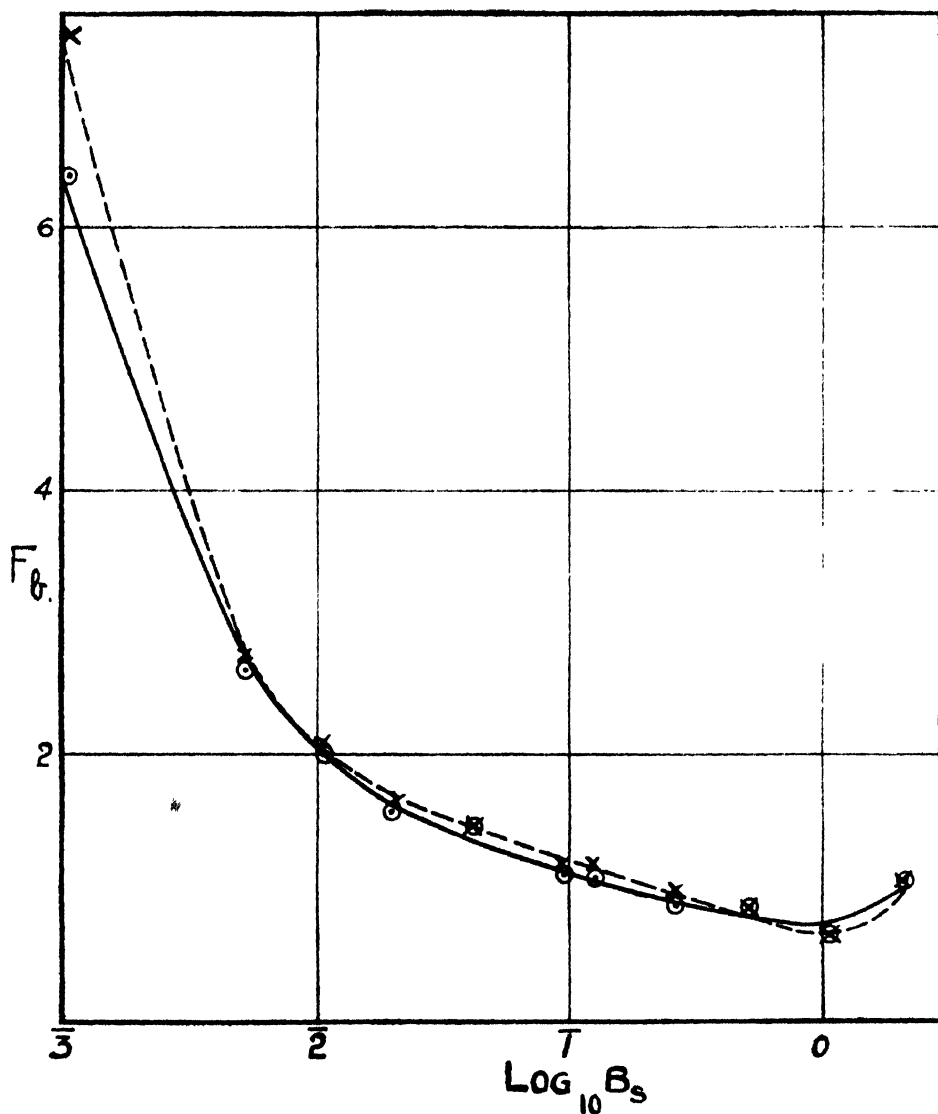


FIG. 8.

Results for a Glare Angle of 3°.

It was decided to make a more extended study of the effect at this angle than considerations of time would allow for all the angles. Five background brightness levels were chosen and for each level five runs were made, the values of B_s approximating closely to the brightness levels chosen. The actual values of the brightnesses are shown in Table III.

Table III.—Values of B_g .

Brightness level.	1.	2.	3.	4.	5.
		candles/sq. ft.	candles/sq. ft.	candles/sq. ft.	candles/sq. ft.
Run 1	Zero	$4.05 \cdot 10^{-3}$	$31.2 \cdot 10^{-3}$	$219 \cdot 10^{-3}$	1.08
„ 2	„	3.98	30.2	209	1.07
„ 3	„	4.16	32.5	211	1.07
„ 4	„	3.98	32.0	219	1.06
„ 5	„	4.07	31.6	217	1.07
Mean	Zero	4.05	31.5	215	1.07

The mean data for the variation of the threshold with the glare illumination for the different brightness levels, are reproduced in Tables IV to VIII and graphs of $T_b = \frac{1}{2}(T_{ba} + T_{bd})$ against E_G are shown in figs. 9 and 10.

Table IV.

θ (angle of glare) = 3° . Background brightness = zero.

E_G .	Subject A.		Subject B.	
	T_b	k_b	T_b	k_b
	$\times 10^{-3}$		$\times 10^{-3}$	
0.030	0.203	0.423	0.217	0.286
0.100	0.975	0.380	0.898	0.272
0.190	1.69	0.363	1.51	0.278
0.300	2.39	0.343	2.06	0.322
0.430	3.06	0.343	2.83	0.351
0.590	3.83	0.392	3.69	0.352
0.760	4.64	0.474	4.56	0.347
0.930	5.98	0.542	5.70	0.372

Table V.

θ (angle of glare) = 3° . *Background brightness = $4.05 \cdot 10^{-3}$ candles per square foot.

—	Subject A.		Subject B.	
	T_b	k_b	T_b	k_b
	$\times 10^{-3}$		$\times 10^{-3}$	
0 Initial	0.139	0.331	0.157	0.417
0 Final	0.157	0.229	0.161	0.319
0 Mean	0.148	0.270	0.159	0.364
0.030	0.541	0.273	0.520	0.259
0.090	1.09	0.239	1.06	0.245
0.140	1.34	0.276	1.28	0.273
0.270	1.91	0.314	1.84	0.348
0.430	2.88	0.346	2.71	0.384
0.580	3.82	0.270	3.72	0.390
0.730	4.72	0.398	4.17	0.369
0.900	5.07	0.417	4.73	0.388

Table VI.

θ (angle of glare) = 3° . Background brightness = $31.5 \cdot 10^{-3}$ candles per square foot.

—	Subject A.		Subject B.	
	T_b	k_b	T_b	k_b
	$\times 10^{-3}$		$\times 10^{-3}$	
0 Initial	0.504	0.180	0.442	0.287
0 Final	0.451	0.160	0.468	0.286
0 Mean	0.477	0.170	0.455	0.286
0.02	0.739	0.127	0.669	0.260
0.07	1.40	0.136	1.23	0.221
0.14	1.98	0.157	1.69	0.237
0.24	2.54	0.205	2.11	0.284
0.40	3.38	0.175	2.71	0.280
0.56	3.90	0.192	3.31	0.463
0.73	4.62	0.297	3.84	0.505
0.91	5.62	0.400	4.48	0.524

Table VII.

θ_a^* (angle of glare) = 3° . Background brightness = $215 \cdot 10^{-3}$ candles per square foot.

—	Subject A.		Subject B.	
E_a .	T_b .	k_b .	T_b .	k_b
	$\times 10^{-3}$		$\times 10^{-3}$	
0 Initial	2.00	0.163	1.67	0.283
0 Final	2.03	0.193	1.67	0.247
0 Mean	2.02	0.178	1.67	0.265
0.025	2.25	0.187	1.92	0.260
0.075	2.60	0.177	2.33	0.280
0.135	2.92	0.175	2.62	0.282
0.230	3.51	0.197	3.16	0.300
0.400	4.72	0.263	4.09	0.371
0.560	5.69	0.306	4.89	0.392
0.720	6.53	0.308	5.89	0.412
0.900	7.53	0.364	6.75	0.421

Table VIII.

Angle of glare = 3° . Background brightness = 1.07 candles per square foot.

	Subject A.		Subject B.	
E_a .	T_b .	k_b .	T_b	k_b
0 Initial	5.67	0.270	5.76	0.297
0 Final	6.22	0.300	5.77	0.336
0 Mean	5.94	0.285	5.76	0.316
0.030	6.04	0.314	6.16	0.343
0.100	6.45	0.305	6.99	0.296
0.200	7.07	0.350	7.82	0.318
0.300	7.77	0.381	8.56	0.258
0.450	8.89	0.399	9.36	0.310
0.630	9.29	0.346	9.70	0.407
0.790	10.30	0.369	11.11	0.468
0.960	11.60	0.396	13.15	0.553

For the two higher levels, curves 4 and 5, a straight line could be drawn to represent adequately the experimental points. For the lower levels, however, the curves are more complex and show a definite curling down towards the origin as the zero glare illumination is approached. The derivation of a formula to give an overall representation of the experimental results is most conveniently obtained by means of a device used by Holladay.

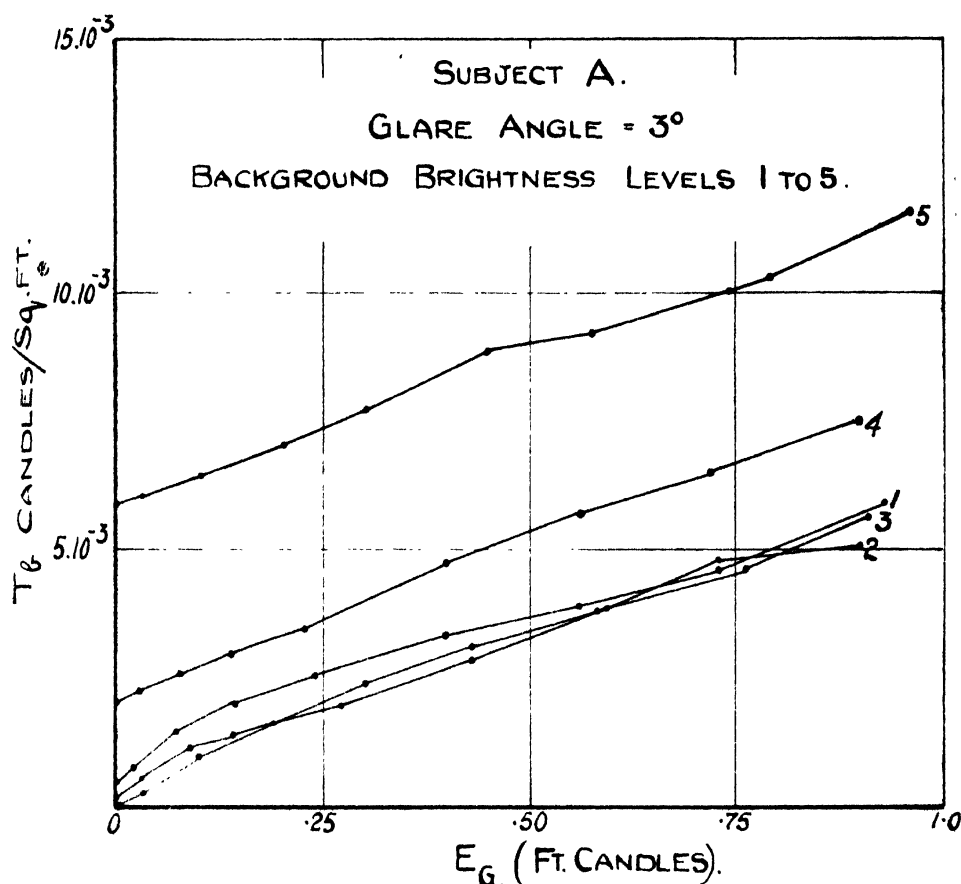


FIG. 9.

Before carrying out this analysis, however, we may draw attention to the following interesting feature of the results. This is best seen by a reference to figs. 11 and 12, in which $\log_{10} T_b$ has been plotted against E_G . For subject A (fig. 9) the curve for the brightness level 2 shows, when compared with the other curves, a definite sag in the E_G range 0.2 to 0.6 foot candles. The same is true, although to a less extent, for the curves of subject B. If this corresponds to a real effect, it means that, at values of E_G in the neighbourhood of 0.4 foot candles, the threshold for a fixed glare illumination does not steadily increase with increase of background brightness, but instead exhibits a minimum at some value of B_s in the neighbourhood of 4×10^{-3} candles per square foot.

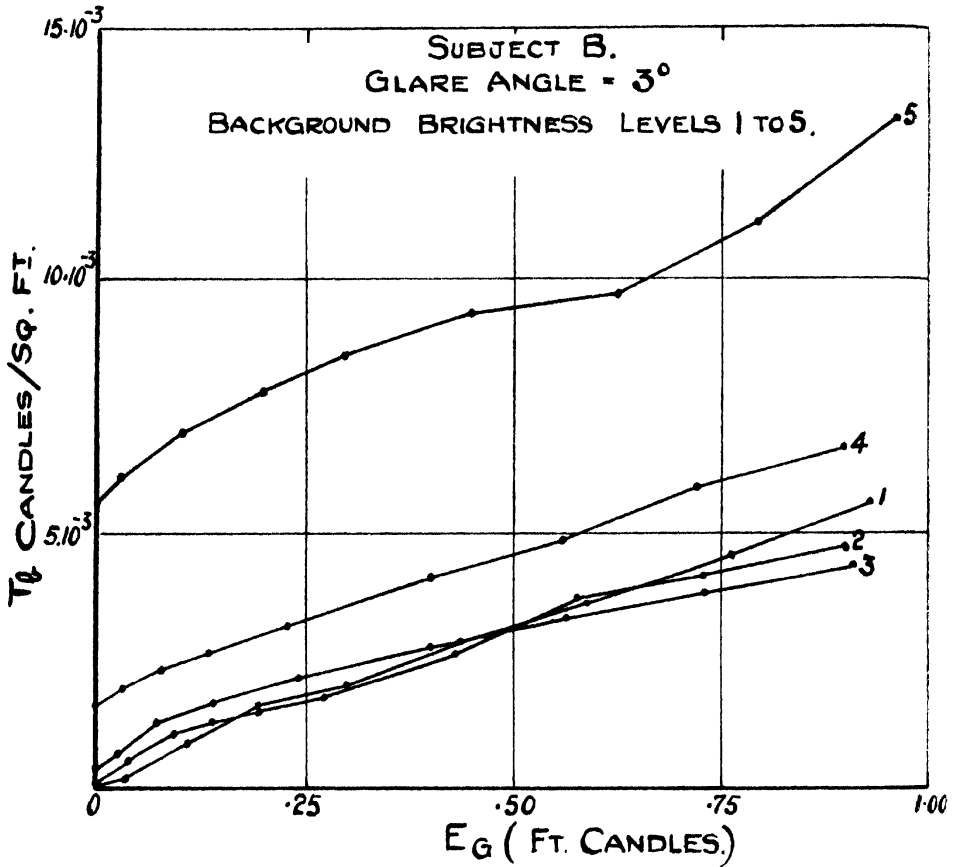


FIG. 10.

A special investigation was undertaken to test this point further. Four runs were made, in all of which the glare illumination at the eye had the same fixed value 0.40 foot candles. Twenty sets of readings were taken in each run, alternate sets being taken at zero background brightness and at one of the remaining background brightnesses. Thus each run gives a comparison of the threshold value under a given glare and background, with the value under the same glare but with zero background brightness. The results are summarised in Table IX.

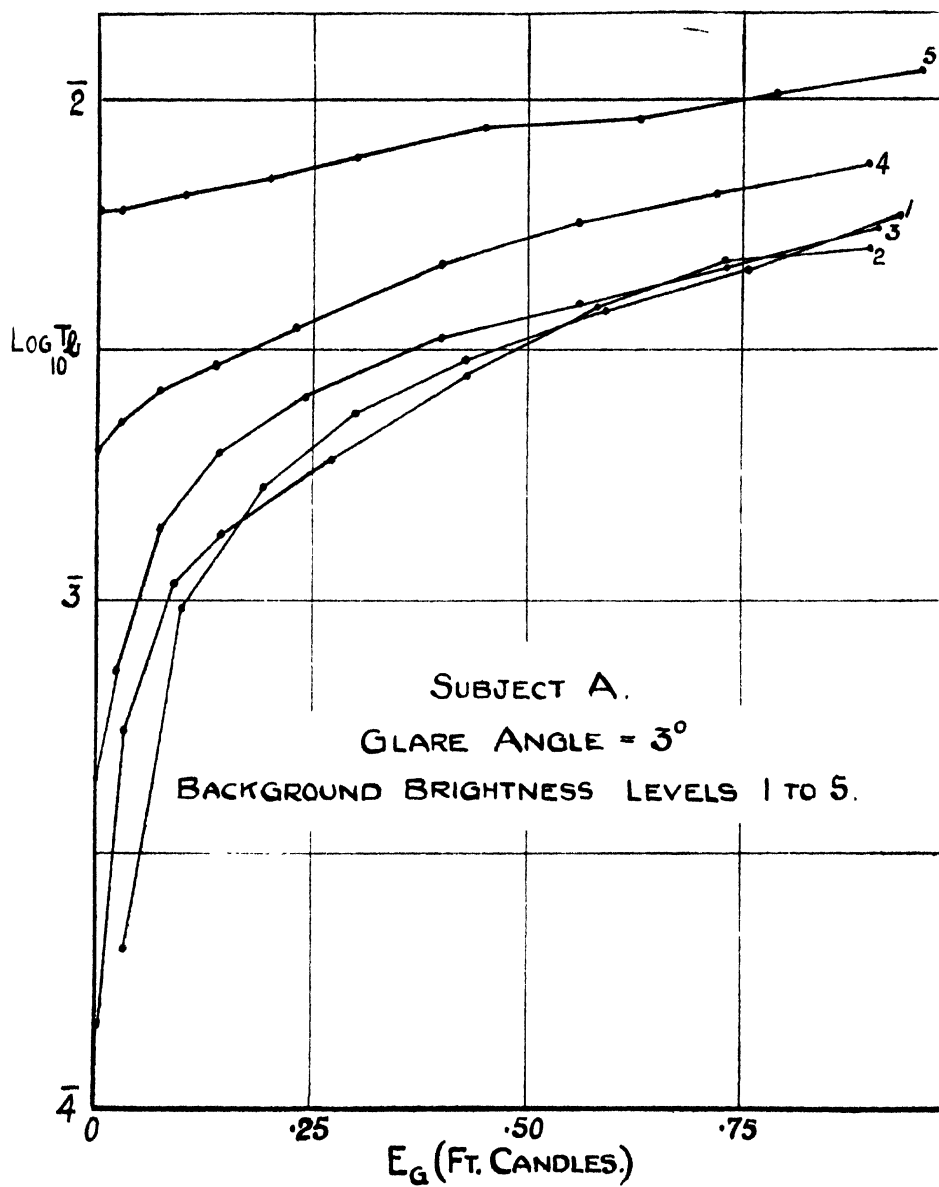


FIG. 11.

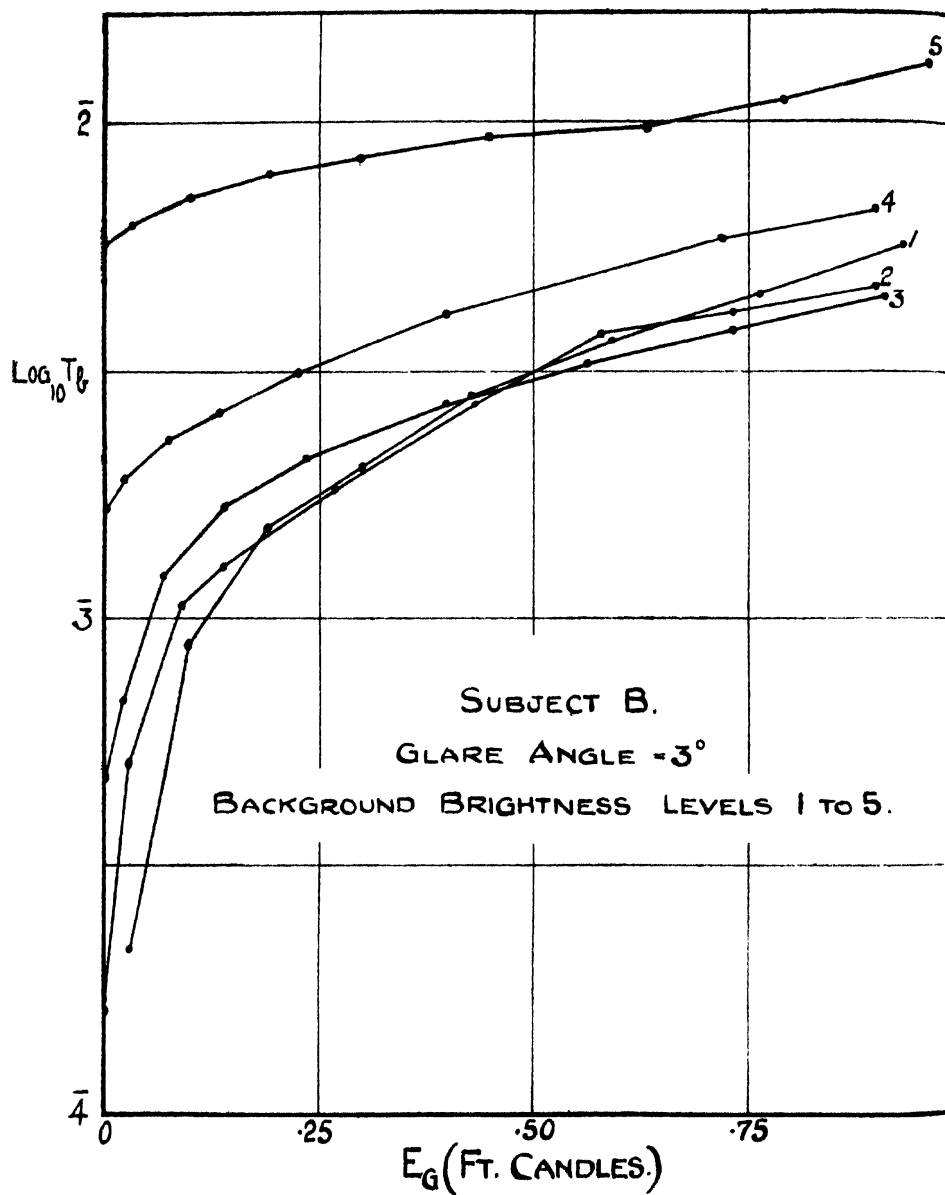


FIG. 12.

Table IX.
Glare angle 3°. $E_G = 0.40$ foot candles.

Background brightness.		T_b Mean of 100 observations.		$(T_b)_{B_g}/(T_b)_0$	
		A.	B.	A.	B.
Run I	$\left\{ \begin{array}{l} 0.00367 \text{ candle/sq. ft.} \\ \text{Zero} \end{array} \right.$	0.00296	0.00298	0.89	0.90
	..	0.00331	0.00331		
Run II	$\left\{ \begin{array}{l} 0.0312 \\ \text{Zero} \end{array} \right.$	0.00334	0.00330	1.02	1.04
	..	0.00329	0.00318		
Run III	$\left\{ \begin{array}{l} 0.209 \\ \text{Zero} \end{array} \right.$	0.00330	0.00378	1.04	1.16
	..	0.00318	0.00326		
Run IV	$\left\{ \begin{array}{l} 1.04 \\ \text{Zero} \end{array} \right.$	0.00723	0.00882	2.14	2.84
	..	0.00338	0.00311		

In fig. 13 the ratio $(T_b)_{B_g}/(T_b)_0$ is plotted against $\log_{10} B_g$. For both subjects the ratio for $B_g = 0.00367$ candles per square foot lies below unity by 10 per

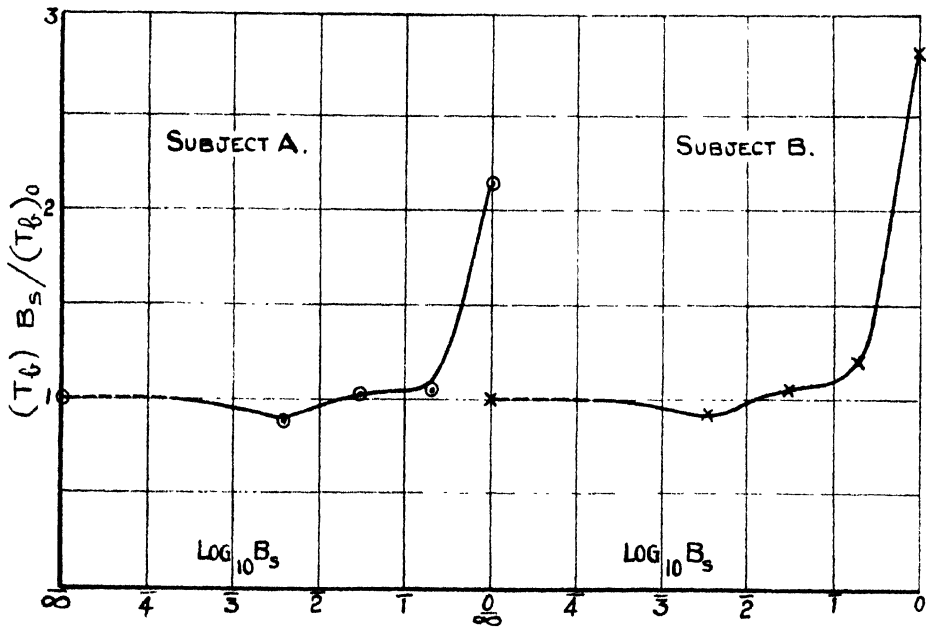


FIG. 13.

cent., an amount which under the conditions of these experiments is larger than can be attributed to experimental error. We may therefore conclude that the special feature of the curves of the main investigation corresponds to a real effect. The magnitude of the effect is however small and in many applications, notably in the derivation of an overall formula, it may be neglected without serious error. From the theoretical standpoint the result obtained is of interest and may be worth following up in a subsequent investigation.

Holladay's method of representing the results of investigation such as these, is to determine the background brightness which alone, *i.e.*, in the absence of the glaring source, would lead to the same value of the threshold as is actually obtained under any specified glare and background conditions. The equivalent background brightness β defined in this way can be obtained immediately for any measured threshold value if the relation between threshold and background brightness in the absence of glare is known. The measurements in the absence of glare (Table II) provide the necessary data and two curves (one for each subject) have been drawn relating T_b to B_s , for values of T_b ranging from about $5 \cdot 10^{-5}$ to $2 \cdot 10^{-2}$ candles per square foot. Using the appropriate curve for each subject the T_b values in the Tables IV to VIII have been read off and the corresponding values of β plotted against E_G for each background brightness level. A typical graph obtained in this way is shown in fig. 14. For all the

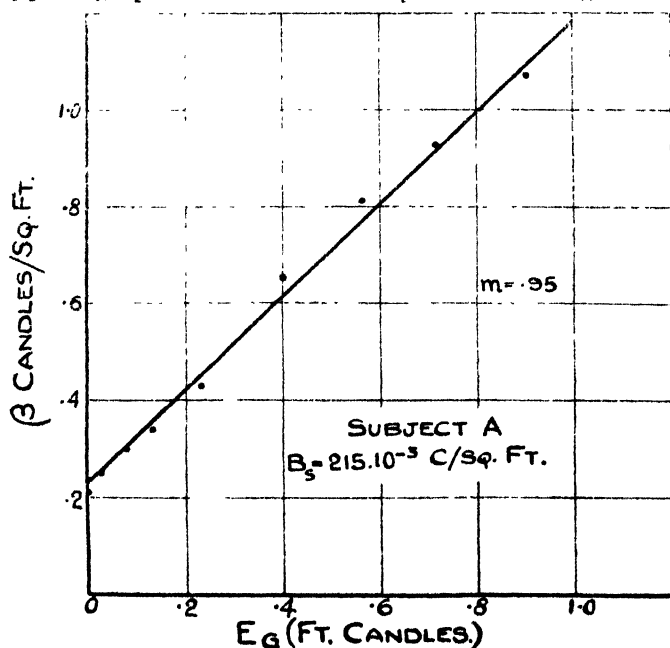


FIG. 14.

background brightnesses the plotted points lie tolerably well on a straight line. The slope m of the best straight line through the points is given in Table X.

Table X.—Glare angle = 3° .

Background brightness B_s candles per square foot	Value of m .	
	Subject A.	Subject B.
0	0.86	0.85
0.00405	0.83	0.735
0.0315	0.82	0.655
0.215	0.95	1.00
1.07	0.67	0.845

Despite the fact that the values of m show variation from 0.65 to 1, it is a tolerable first approximation to put m equal to a constant independent of background brightness. If this is done, the results calculated agree with those observed within the tolerance which it is necessary to allow in work of this kind. The results at 3° conform therefore to a formula of the type

$$\beta = B_s + m(0) E_G,$$

or the effective background brightness equals the actual background brightness B_s plus a term independent of B_s , and proportional to E_G the glare illumination at the eye.

Results for other Angles.

For the other angles, 1° , 2° , 5° and 10° , two runs were made at each background brightness level. The results of each run were used to obtain a β curve as above. The β curves obtained in this way being based on one run only were not so regular as those for 3° based on five runs. In every case, however, the best straight line was drawn through the plotted points. The mean m value for the four runs (two for each subject) is shown in Table XI, where the means of the m value for $\theta = 3^\circ$ are also included.

Table XI.—Values of m .

Angle θ .	Background Brightness.					Means 1 to 4 inclusive.
	1. Zero.	2. 0.00416.	3. 0.0376.	4. 0.222.	5. 1.10.	
1°	3.24	3.62	3.34	2.94	2.22	3.29
2	1.78	1.82	1.84	1.69	1.96	1.78
3	0.86	0.78	0.74	0.98	0.74	0.84
5	0.414	0.252	0.296	0.446	0.634	0.352
10	0.125	0.080	0.143	0.125	0.638	0.118

The data of this table are shown graphically in fig. 15 where $\log_{10} m$ is plotted against $\log_{10} B_s$ for each angle. It will be seen that although for 2° and

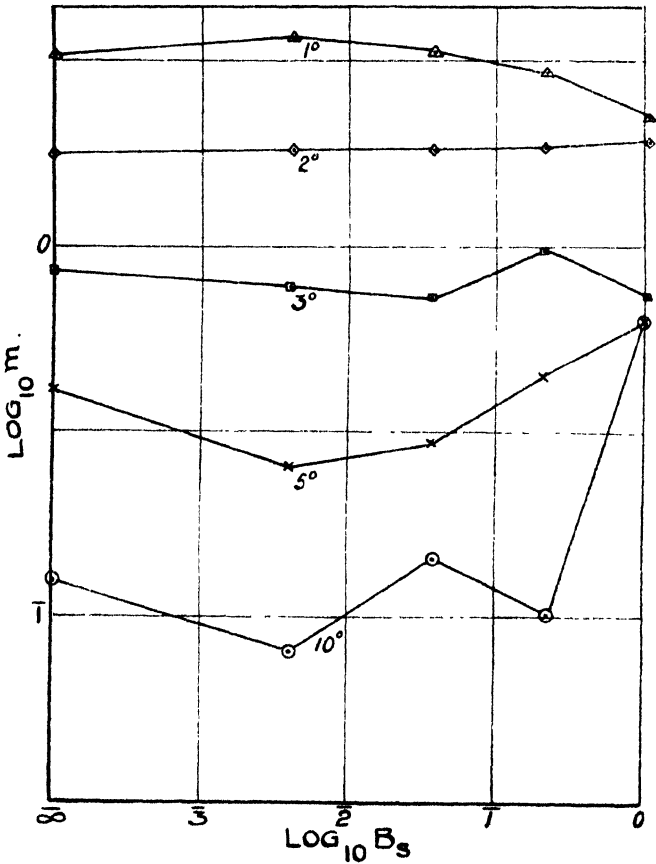


FIG. 15.

3° and to a less extent for 1° the value of m is little changed in passing from zero background brightness to $B_s = 1$ candle per square foot, for the other angles marked variation occurs, particularly at the highest brightness level. The most extreme case is for $\theta = 10^\circ$ for which m at $B_s = 1$ is five times m at $B_s = 0$. It should be borne in mind in considering these results that for $\theta = 5^\circ$ and $\theta = 10^\circ$ the measurements of m for the highest brightness level are subject to greater error than the others. At the highest brightness level the threshold is determined chiefly by the background brightness and the difference between no-glare and maximum glare measurements is small. The error in determining m is correspondingly large.

It appears, however, unlikely that the increased value of m at the highest background brightness is attributable entirely to this cause. Suppose for each background m is of the form k/θ^n where k and n may vary with B_s . The best values of n to fit the data of Table XI are plotted against the corresponding values of $\log_{10} B_s$ in fig. 16. It appears from this that the value of

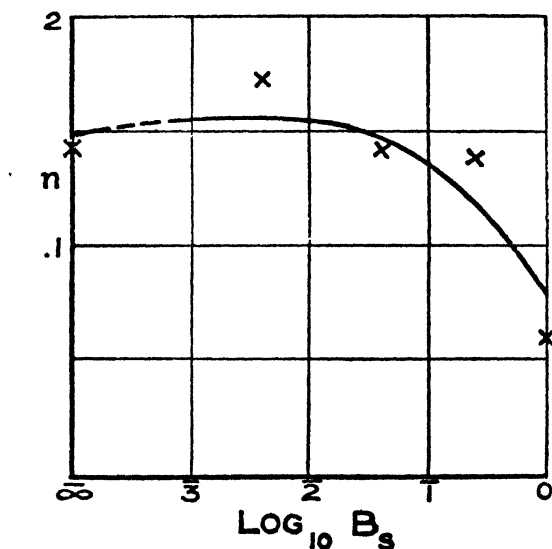


FIG. 16.

m is practically independent of B_s and equal approximately to 1.5, for values of B_s not exceeding 0.22 candle per square foot, but for higher background brightnesses the law changes. An overall formula to represent the m values for $B_s \leq 0.22$ candle per square foot is best obtained using the mean m 's of column 7, Table XI. The result obtained is $m = 4.16/\theta^{1.5}$. Thus

$$\beta = B_s + \frac{4.16}{\theta^{1.5}} E_G.$$

350 *Effect of Glare on Brightness Difference Threshold.*

This may be compared with Holladay's expression for β , which when transformed into the present units, runs

$$\beta = B_s + \frac{9.2}{\theta^2} E_G.$$

At $\theta = 3^\circ$, the expressions do not differ greatly for $\frac{9.2}{\theta^2} \triangleq 1$ and $\frac{4.16}{\theta^{1.5}} \triangleq 0.8$.

The present formula compared with Holladay's indicates less effect due to glare for angles less than 3° and more effect for angles greater than 3° .

It will be noted that keeping E_G and θ constant the formula makes β and therefore T_b an increasing function of B_s . The observations recorded on p. 345 show that this is not always in accord with experiment. In fact, the formula suppresses the special effect to which attention has been drawn above. This does not detract from its usefulness as giving a good overall representation of the results.

Summary.

A method is described for determining the brightness-difference threshold in presence of a point-source of glare. The percentage probable error for a single observation of the threshold is found to equal approximately 7 per cent. In addition the results indicate that the threshold is subject to fluctuation during an extended series of readings, particularly in the case of inexperienced subjects.

The value, for two subjects, of Fechner's Fraction in absence of glare has been found for field brightnesses ranging from 0.001 to 2.2 candles/sq. ft. The threshold in the presence of the glare source is best expressed in terms of the equivalent background brightness β . The following formula is established for β and represents the results tolerably well over the range indicated :—

$$\beta = B_s + \frac{4.16}{\theta^{3/2}} E_G,$$

$$0 < B_s < 0.22 \text{ candles/sq. ft.}, \quad 0 < E_G < 1 \text{ ft. candle},$$

$$1^\circ < \theta < 10^\circ.$$

For background brightnesses greater than 0.22 the formula breaks down.

A systematic deviation from the formula (and from any formula which makes β an increasing function of B_s under any glare condition) occurs for $E_G = 0.40$ and $\theta = 3^\circ$. The threshold with these values of E_G and θ is about 10 per cent. greater for $B_s = 0$ than for $B_s = 0.0037$ candles/sq. ft. This effect is not great enough to interfere seriously with the formula.

The writer has much pleasure in expressing his appreciation and thanks for the assistance given him by Messrs. B. J. Oram, G. C. Cornford, G. E. V. Lambert and C. J. Macmanus, who acted as subjects in the investigation.

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The Effect of X-Radiation on the Spermatogenesis of the Guinea-Pig.

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[PLATES 17–20.]

Introduction.

The effect of X-rays on tissues and cells has been the subject of study by many observers, dating back to the time when Leopold Freund, of Vienna, burnt the first patient. We were led to study this problem merely from the hope that the more modern cytological techniques might reveal some new facts with reference to the mystery of the effect of X-rays on the cell. Moreover, we hoped to collect some interesting facts about the mechanism of sperm formation.

We are grateful to the British Medical Association for a grant in aid of this work; to Dr. Bethel Solomons, the Master, and Dr. M. MacDonagh, the Radiologist of the Rotunda Hospital, for the use of the hospital X-ray plant and laboratory, as well as for their constant interest and encouragement, and to many people for the loan of literature. One of us (S.W.) has received a Research Scholarship from the Irish Free State Government, whom we most cordially thank.

Previous Work.

There are a large number of researches carried out on X-ray sterility in the testis. Albers-Schönberg in 1903 first studied the effect of X-rays on the testes of guinea-pigs and rabbits, showing that while no loss of potency was

caused, no offspring were produced when X-radiated males were paired with normal females.

The persistence and overgrowth of the Sertoli cells was established by Bergonié and Tribondeau in 1904, who worked with the rat. Wakelin Barrat and Arnold in 1911 carried out a more elaborate investigation into the effect of X-radiation on the rat testis. We have obtained the following résumé of their work from Colwell and Russ, "Radium, X-rays and the Living Cell," as we have been unable to obtain a copy of the original paper :—

The tube was of medium hardness, the current from 0·6 to 0·7 milliamperes, and a 15-minute exposure to the rays gave ordinarily a reading of one unit on the scale of Sabouraud.

Spermatogonia.—After a moderate exposure these cells persist for about three days, but after the fourth day they cease to be observable. There is a total absence of mitosis during the time these cells are visible.

First Spermatocytes.—During the first few days following exposure, these cells develop marked changes, and owing to their large numbers and prominent characters their alteration is responsible for a great deal of the changed appearance of the seminiferous tubule. The alterations are of two kinds, necrotic and non-necrotic.

(a) *Necrotic*.—Three days after exposure large necrotic masses appear, but disappear after the ninth day. Early necrosis is heralded by contraction of the nucleus, disappearance of the nuclear membrane, and poor staining of the spireme.

(b) *Non-necrotic*.—These are various and include enlargement of the clear space surrounding the intranuclear body, formation of intranuclear vacuoles, and thinning of the spireme. Multipolar mitoses occur 24 hours after exposure, spindles varying from two to four.

Second Spermatocytes.—These cells are relatively inconspicuous and few in number (*sic*, Colwell and Russ, p. 272) and so are difficult to observe. They disappear about five days after exposure, but their method of disappearance is uncertain. Amitosis occurs.

Spermatids.—Young spermatids may undergo necrosis either before or during formation of the thread. This happens especially four days after exposure. Von Ebner's granules appear sooner.

Spermatozoa.—The only change appears to be softening, as shown by separation of filaments.

Abnormal Cells due to X-rays.—These are multi-nucleate and multi-vacuolate cells.

The subject of X-ray sterility has been investigated in France, especially by Regaud. This worker has viewed the subject more from the histological aspect than we have done. He insists that the spermatogonia are the most radio-sensitive of all the elements of the testis, and that their cytolysis may be found two hours after irradiation. As the purpose of the present paper is cytological rather than histological, and as Regaud has used the rat principally, we do not propose to deal with his histological findings at present. In another paper one of us (S.W.) will consider Regaud's findings, in the light of our own material made from irradiated rat testis.

Perthes, in 1903, was the first observer to point out the relationship between activity of reproduction in tissues, and degree of radio-sensitivity. He came to this conclusion by his investigations on the effects of X-radiation on developing eggs.

In 1906, Regaud and Blanc, working on seminal tissue, came to the conclusion that the period of mitosis was the most sensitive, and in the same year Bergonié and Tribondeau formulated the following "law":—"Les rayons X, agissent avec d'autant plus d'intensité sur les cellules que l'activité reproductrice de ces cellules est plus grande, que leur devenir karyokinétique est plus long, que leur morphologie et leurs fonctions sont moins définitivement fixées."

Turning now to some other investigations, not on spermatogenesis, but on cell division, we may refer especially to the results of Strangeways and Hopwood (26); their conclusions, obtained from a study of X-radiated tissue cultures, were as follows:—

1. The earliest recognisable effect of X-radiation upon growing cells is the temporary inhibition of the onset of mitotic division in the majority of those fully formed vegetative cells which are about to divide.
2. Cells actually undergoing mitosis are unaffected by X-radiation.
3. It appears that vegetative cells pass through a phase during which the physiological processes of the cell are especially liable to be disturbed: if such a cell receives a dose of X-rays greater than 30 c (about 170 R according to our system of measurement) it will nevertheless enter mitosis, but the process of division will be of an abnormal type and may result in complete disruption of the cell.
4. No evidence was obtained of any stimulating effect of X-rays.
5. Softer rays appear to be more efficient.

One of us (J. B. G.) has had the opportunity of seeing the late Dr. Strangeways' methods and material, and considers his results of great importance in any consideration of the effects of X-radiation on cells. This work will be considered further in the discussion.

More recently Alberti and Politzer have studied the effects of radiation on mitosis in cultures of salamander larvæ, and describe three stages in which radiation effects are manifest (Levitt, 18). First, there occurred a period during which marked degenerative changes were seen in the nuclei of the irradiated cells, terminating in complete cessation of mitosis. This period lasted for 6 hours, and was unaffected by the size of the dose. In the second stage mitosis was completely absent. The duration of this stage did vary with the size of the dose. With a moderate dose (about 80 per cent. of the human skin dose) it was 3 days. At the beginning of the third stage mitosis recommenced, but showed marked abnormalities, such as incomplete migration of the chromosomes along the spindle, producing "gummy" chromosomes, as first shown by Perthes.

J. A. Crowther (9), by a series of mathematical formulæ, has come to the conclusion, following Strangeways and Oakley's results on X-radiation of chick embryo cultures, that the cellular structure which is hit by the rays might be smaller than the cell itself, as it is indicated by the author's calculations that, assuming that the particles hit are spherical and have unit density, the size to account numerically for the observations on the theory suggested would be 4×10^{-5} c.mm., or $1/2500$ mm.

We are unable to assess the value of Crowther's views, or of those expressed in his later paper ('Proc. Camb. Phil. Soc.', vol. 23 (1926)). In this contribution he calculates that the protozoon, *Colpidium colpoda*, needs 49 hits in some particular structure before it is killed by X-radiation. Crowther gives no data as to what proportion of his protozoa were dividing, whether it was such individuals which were killed, and whether he used controls.

Technique and Material.

The French workers Bergonié and Tribondeau used as control a testis removed just before X-radiation. Thus, added to the X-radiation effect was shock, and the use of an anæsthetic. We are well aware of the deleterious effects of ether or chloroform on cells, and have avoided this technique. The only safe method is to make a proper study of normal testis, and to repeat the experiments with X-rays several times. Animals which were sick, or dying, were discarded, and such material has not been taken into consideration.

In normal testes many abnormalities may occur. Years of experience in spermatogenesis studies has helped one of us to distinguish between normal necrotic effects and those produced by X-rays. It has been necessary to be able to distinguish between loss of position in the spermatic wall, genuinely caused by X-rays, and that caused by the scissors and knife used in dissecting out the material. All such possible sources of deception have been noted, as well as our experience has enabled us.

The techniques used by us were Kolatchev, Da Fano, Regaud, and Bouin. The three last are practically useless, and most of our results are based on some of the really beautiful Kolatchev preparations which one of us (S. W.) has made. The objection to the Da Fano and Regaud material is that it is not uniform enough. Bouin, of course, gives tolerably uniform results, but it is not in the same class as the Kolatchev method.

The advantage of the Kolatchev method is that one gets a good fixation with Champy's fluid, and then a thorough hardening in OsO_4 . Variations in dehydration, dealcoholisation, and embedding hardly show, and cytologically the method is the best known to our science. The Kolatchev sections were counterstained with neutral-red acetic, as directed by R. Ludford (11).

We chose the guinea-pig because its spermatozoon is very like that of the human, whereas that of the rat is quite different.

Outline of Conditions of Application.

Apparatus.—Snook transformer.

Tube.—Metalix water-cooled, type E. (This is a type of Coolidge tube.)

Voltage.—90 kv. (soft rays) or 120 kv. (hard rays).

Spark-gap.— $5\frac{1}{2}$ or $7\frac{3}{4}$ inches between point and plate.

Amperage.—2 milliamps. *Distance.*—37 cm.

Dosage.—250 R—10,000 R., i.e., $\frac{1}{4}$ to 10 pastille doses.

Time.—For one pastille dose at 90 kv., 20 minutes; at 120 kv., 15 minutes.

All the hair over the testis was completely cut away, and the guinea-pig was placed on its back and spread-eagled out on a wooden base board. The testes which had retracted into the abdomen were pushed down until they filled the scrotal sac, and a tight pad and binder was placed round its abdomen to prevent further retraction. A sheet of lead, in which a hole had been cut to correspond with the testes, was then placed over the animal, so that with this exception it was completely protected from X-rays. The lead was raised off the head by means of wooden blocks, and then slanted down until it was touching the abdomen.

Personal Investigations.

For the purpose of convenience we may divide our examples into four classes :

1. Normal material used as controls.
2. Material X-radiated sufficiently to produce mild changes, such as inability of a certain number of cells to mitote.
3. Material X-radiated sufficiently to produce extensive degeneration not followed by growth of Sertoli-cells, *i.e.*, in which recovery is always possible.
4. Material so intensely X-radiated as to produce more extensive destruction followed by growth of Sertoli-cells, and consequently choking of at least the majority of the spermatid tubules, *i.e.*, in which sterility is usually effected.

We have procured many examples which are intermediate between these four classes. Taking the X-radiated material according to the three latter classes above mentioned, we may describe the broad histological appearances as follows :—

In Class 2, a few spermatogonia are killed during mitosis, and many secondary spermatocytes fail to undergo second maturation division, though they live for many days. The general arrangement of the tubes is little altered, and active mitosis may occur in all stages of spermatogenesis. Multi-nucleate cells occur, but are rare and non-necrotic.

In Class 3, many multi-nucleate cells occur, and giant cells are formed by complete coalescence of nuclei and cytoplasm of two or three cells. Many spermatocytes, unable to undergo mitosis, but alive and otherwise healthy, occur. Disappearance of the oncoming cells occurs, and tends to alter the appearance of certain tubes. Tubules depleted of all cells except spermatogonia Sertoli-cells and ripe spermatozoa are common.

In Class 4, the tubules are depleted except for a few necrotic pre-spermatid and spermatid cells, and the Sertoli cells have grown strongly into the empty tubules.

Class 1. Normal Material.—A large number of control slides from different animals have been examined, and the example shown on Plate 17, fig. 1, has been drawn as typical. The lumen of the spermatid tubule is on the right : outside the tubule, in the bottom left at INT. T., are interstitial cells. The lining cells of the tubule are of two kinds, spermatogonia, SGN., and Sertoli cells, SER. The resting spermatogonia are flattened cells, whose nuclei

contain nuclear canals, as is usual in vertebrate spermatogonia. These nuclear canals penetrate well into the centre of the nuclei, and, when cut across, give the illusion of amitosis. All the resting spermatogonia contain granules of neutral fat, mitochondria and an eccentrically placed Golgi apparatus.

These cells are the mother or stem cells of the line of cells leading to the ripe spermatozoa. At G. SGN. is a spermatogonium passing towards the prophase of mitosis. After mitosis the inner cell remains adherent to the spermatic tubule wall, the outer sister cell continues to grow, and enters the prophase of the heterotypic division and may be known as a growing spermatocyte. Such cells are drawn at YSP., and form the next layer of cells in the spermatic tubule wall. When the prophases are drawing to a close, the full-grown spermatocyte is found to be a cell, such as is drawn at 1st SPY. in Plate 17, fig. 1, and shown in the microphoto, fig. 9, in Plate 20. The primary spermatocytes contain a large Golgi apparatus, numerous mitochondria, a vacuolar system, a chromatoid body, and the primordia of the post-nuclear bodies of the ripe spermatozoon; these structures have all been mentioned in a previous paper (10).

When ripe, the first spermatocytes divide and give rise to two daughter second spermatocytes, shown in fig. 1 as 2nd SPY. These cells are twice as numerous as the primary spermatocytes, but divide very soon to form spermatids, and as the primary spermatocytes have a long growth period they appear more numerous in certain parts of the tubules.

The secondary spermatocytes have a nucleus and Golgi apparatus as shown in the diagram in fig. 1 at 2nd SPY. The nucleus contains two or three chromatic bodies, and the nucleoplasm has become denser. Such cells, as have been mentioned, quickly undergo a second mitosis and produce smaller cells closely resembling the second spermatocytes. These are the spermatids, and as they undergo a long series of changes, the spermatids, like the first spermatocytes, almost always appear numerous at any given part of the tubules. Such cells are shown at STD. in fig. 1. In the early spermatid the Golgi apparatus and mitochondria exactly resemble those of the earlier generations of cells, except for the smaller size of the former, and the smaller number of the latter.

We then come to the metamorphosis of the spermatid into the spermatozoon. These changes have been dealt with in previous papers, and only those of special interest to the present investigation will be mentioned. In Plate 17, fig. 1, at STD., the previously rounded Golgi apparatus (STD. G., in a cell more to the right) has moved up and partially flattened on one side, overlying

the nucleus. Presently a grey cap, the acrosome, becomes formed on the nucleus, as shown in Plate 19, fig. 3, and this cap becomes differentiated into inner and outer parts.

The granules within the nucleus gradually break up and disappear, and a more reticular arrangement supervenes, as shown in fig. 8 (Plate 19). The Golgi apparatus slips down away from the front of the nucleus, and the acrosome becomes a hemispherical body on the nucleus. Further stages need not detain us here, but are shown in Plate 18, fig. 2, lower right, next stage Plate 17, fig. 1, upper right, next Plate 18, fig. 2, upper right, which are practically ripe. The Golgi apparatus passes down into the cytoplasm remnant (STD. G.), and Von Ebner's granules are produced by fatty degeneration of many of the mitochondria, V.E.B., in figs. 1 and 2. The reader is referred to the previous publications of the writers on the subject of the fate of the various cell elements during these remarkable changes (10, 13, 14).

As the suggestion has been made (9), that the centrosome may be the cell body injured most by X-radiation, it is of some interest to give some notes on its behaviour and position during spermatogenesis in *Cavia*. In the growing spermatogonium and spermatocytes, the centrosome lies inside the Golgi-idiozome complex; just before the first maturation division it comes out of the idiozome or archoplasm, and lies towards the nucleus. After the first maturation division the centrosome does not become embedded in the second spermatocyte archoplasm, but lies outside, and keeps outside henceforth. In the early spermatid the centrosome forms two parts, a head bead and a middle piece ring. The main point to be noted here is that the centrosome of the second spermatocyte and spermatid never enters the archoplasm-Golgi complex.

Now, for the purpose of comparison, and later of discussion, the main events in spermatogenesis may be summarised as follows:—

- (a) There are three periods when mitosis occurs. In the spermatogonia, and in the first and second spermatocytes.
- (b) There is a period of growth and of complicated movements of chromatinic filaments, known as the prophases of the heterotypic division.
- (c) There is a long period of the metamorphosis of spermatid into spermatozoon, during which the nucleus mitochondria, Golgi body and centrosome undergo remarkable evolutions or changes.

The structures implicated more or less directly in these changes are:—

- (1) Chromosomes; (2) nucleolus; (3) centrosome; (4) Golgi complex;
- (5) vacuolar system; (6) post-nuclear bodies; (7) chromatoid body.

It has been our purpose to endeavour to follow out the behaviour of each of these structures under X-radiation. Previous workers have suggested that the X-radiation causes some deleterious change in :—

(a) The nucleus and chromosomes ; (b) the centrosome ; (c) the lecithin content of the cell ; (d) the colloidal condition of a part or parts of the cell.

Class 2. Material X-radiated sufficiently to produce mild changes.—The specimen from which Plate 18, fig. 2, was drawn was irradiated with 1 pastille dose of hard rays, or 1000 R at 120 kv. for 15 minutes, in one application, and examined 4 days afterwards.

On Plate 18, fig. 2, at S. SGN., is a dead spermatogonium which has undergone pycnotic changes. Such specimens are rare, and cannot be taken as especially characteristic. We mention this because, contrary to the view of Regaud, we find the spermatogonia very resistant. The ripening spermatozoa on the right of the tubule projecting into the lumen, are at a different stage of development from those in fig. 1. This signifies nothing, and is not meant to indicate difference between the two specimens.

The most characteristic elements are the secondary spermatocytes at 2nd SPY. These are of normal size, their nuclei seem quite typical, but in each example the acrosome has been secreted. Another such cell is shown above at 2nd SPY., and below, lettered similarly. In the lower example the growing acrosome is at A. Now in Plate 20, fig. 10, two such cells are shown at 2nd SPY., and also one in fig. 12, the latter showing the acrosome (fig. 12, *bis*). In Plate 19, fig. 3, a normal spermatid, and in fig. 4, a secondary spermatocyte with acrosome (A) are shown.

Turning again to Plate 18, fig. 2, at AN. 1st SPY., we find a peculiar type of first spermatocyte with a nucleus like that of a spermatid. Such cells are fairly common. A normal looking spermatocyte is shown at N. 1st SPY. Many quite normal spermatocytes, and many apparently normal mitoses of both spermatogonial and spermatocyte stages occur. Now in the material thus irradiated many spermatocytes of both orders are unable to undergo mitosis, but normal acrosome formation occurs, especially in the secondary spermatocytes. In this manner "giant spermatids" are produced.

Class 3. Material X-radiated so as to produce greater changes leading to the production of giant-cells by actual coalescence, but no noticeable outgrowth of Sertoli cells. The example from which Plate 19, figs. 5, 6 and 7, was drawn received the following dosage : $5\frac{1}{2}$ pastille doses of hard rays, in 9 applications as follows : 16.8.28, 500 R at 120 kv. in 8 minutes or $\frac{1}{2}$ pastille dose : 30.8.28,

2500 R at 120 kv. in 38 minutes, or $2\frac{1}{2}$ pastille doses. Remainder of doses given in two applications daily.

This type of treatment produces many abnormalities in the spermatids, at the time when the acrosome is hemispherical in outline, or at the stage shown in Plate 19, fig. 8, normal cell. In such material giant cells,* as shown in Plate 19, fig. 6, are produced. This has been formed by the coalescence of three spermatids, whose three centrosomes and three tails are fixed in different positions on the nucleus at C. The nucleus is elongating normally, though the acrosome is not in its correct position. The three Golgi bodies fused to form a single large structure at G. The other parts of this cell will be described below.

In Plate 19, fig. 7 is a common type of cell, formed by the coalescence of two nuclei. In this case the Golgi bodies (G) have kept apart, but each has contributed to form a common acrosome (A). The two centrosomes (C) have become fixed and have grown out to form two tails. In this material many pycnotic nuclei are found, though mitosis occurs in both groups of cells (spermatogonia and spermatocytes).

Class 4.—This material was useless for cytological study, as it merely showed the invading Sertoli cells. The dosage here was mixed, given at both voltages of 120 kv. and 90 kv. until a very large dose was given and the skin burnt.

GENERAL CYTOLOGY.

The Golgi Apparatus.

The Golgi apparatus complex of the Cavy has been much studied, both *intra vitam* by Hirschler, Monné, Brambell, Subba Rau, and the senior writer,

* That such giant cells and polynucleate cells are formed by coalescence and not by an imperfect mitosis, such as produces the apyrene spermatids of certain Lepidoptera and Mollusca, can be proved as follows :—

(a) Actual coalescence of spermatocytes and spermatids, both cytoplasm and nucleus, may be watched happening in smears of testis.

(b) The nuclei of these binucleate and tri-nucleate cells are not one-half or one-third the size of the spermatocyte nucleus or spermatid nucleus respectively, but are actually full size.

(c) The Golgi bodies of such binucleate and tri-nucleate cells are multiple, according to the number of nuclei, and not half or one-third the proper size, as they would be if formed by imperfect division.

(d) The nuclei are never variable in size within the same giant cell, as occurs in apyrene examples of Lepidoptera and Mollusca, where two or more nuclei are formed from variable numbers of chromosomes.

and by many older workers who used the fixing and staining methods. Its structure is thoroughly well known. There is a lipid cortex, which goes black in osmium tetroxide and silver nitrate, and beneath a proteid sphere or idiozome, containing (in the spermatocyte and spermatid stages) a number of vacuoles of another proteid (?) which eventually forms the cap of the spermatozoon.

In Plate 20, fig. 9, the nuclei and clearly marked Golgi bodies of a number of spermatocytes are shown. During the formation of the head cap or acrosome, the pro-acrosomic granules or vacuoles run together to produce the greyish staining acrosome, which soon becomes differentiated into two parts, an inner and an outer (Plate 19, fig. 3, A1, AO).

Now we have shown in this work that this process of acrosome formation does not seem to be affected by X-radiation in so far as concerns its normal course, though not its normal time. In Plate 19, figs. 4, 6 and 7, are giant cells, in all of which the acrosome has been formed despite the abnormality of other parts of the cell. In Plate 19, fig. 5, is a less normal cell, but this had been more vigorously X-radiated and came from a very necrotic area of the testis.

In a number of our X-radiated specimens, the Golgi bodies have a peculiar frayed or granular appearance, which we at first thought might be due to the direct effects of X-radiation. We have been unable to demonstrate this effect constantly in all those experiments especially designed to produce this result, but we believe that it is possible that X-rays do alter the lipid cortex of the Golgi-archoplasm system.

Lag-effect in Mitosis.

It has been mentioned in a previous communication by the senior writer and Ludford (12) that during the prophases of mitosis the Golgi bodies break up into small elements, which become scattered in the cytoplasm, and which are reorganised as spherical structures at telophase. Thus at the end of mitosis, when the second spermatocyte or spermatid nucleus has formed, the Golgi body has also become spherical, and is *in situ* beside the nucleus.

Now in many divisions a distinct lag in this process may be seen. In Plate 18, fig. 2, at CN, upper, are two spermatids with fully-formed nuclei, but in each case the Golgi elements have failed to assemble. In Plate 20, fig. 14, at G¹ and G², are microphotos illustrating the same phenomenon.

Lag-effect in Spermateleosis.

In both the guinea-pig and the insect *Lepisma*, we have noticed lag-effect during the time the spermatozoon is forming. In *Lepisma* (which has been studied by R. N. Mukerji in this laboratory, with the assistance of S. W.), lag-effect is very remarkable. The tail of the spermatozoon goes on elongating in a normal way, but the nucleus does not keep up. The head centrosome fails to make contact with the nucleus, and the latter instead of elongating, remains spherical. In no mammal is this phenomenon so clear as in *Lepisma*, and we mention this investigation by Mukerji, because it is pertinent to the discussion which will follow.

Lag-effect is well known in the development of eggs, such as those of *Ascaris* studied by Perthes, both by radium and X-rays, and seems a common effect.

Radio-sensitivity.

Regaud as above mentioned has claimed that the most radio-sensitive of the testicular elements are the spermatogonia. We find them the most resistant. It is quite true that the neutral-red test shows dead cells belonging to the spermatogonial generation first of all. This test consists of teasing up small pieces of X-radiated testis in neutral-red Ringer. The dead cells then stain bright red in both nucleus and cytoplasm, and stand out clearly. Now in testis irradiated three days before, the dead spermatogonia are not numerous, when we remember that, except for Sertoli cells, the inner walls of the spermatid tubules are paved with flat spermatogonia.

In Plate 20, fig. 13, is a testis which has received a series of severe irradiations, so that no outwardly normal cells except ripe sperms and spermatogonia are left. At SGN are normal spermatogonia,* which could provide new generations of cells. At NS are necrotic second spermatocytes, and at NSPY is a necrotic first spermatocyte. At NSGN is a spermatogonium which has been destroyed. Even though normal spermatogonia may be the last element to "support life" in a severely X-radiated testis, these are usually choked by an overgrowth of the Sertoli cells, which ultimately bring about blockage of the tubes and consequent complete sterility.

* These are not Sertoli cells, because both nuclear and cytoplasmic characteristics are spermatogonial. The Golgi bodies of Sertoli cells are elongate structures, the spherical shape being very rare.

The Vacuolar System.

The vacuolar system of the spermatocyte, and spermatid, is a group of granules (or vacuoles), as shown in Plate 19, fig. 3, V, and in fig. 8, V, for normal cells. In all stages the vacuoles hover near the Golgi body.

On the same plate, in figs. 4, 5, 6 and 7, we see that the vacuolar system keeps to its normal position, and is not visibly altered by X-radiation. Fig. 7 is a particularly interesting specimen, because it has been formed by the union of two immature spermatids (before the stage of fig. 3), and these two have constituted a peculiar cell, with a diploid nucleus, with two Golgi bodies with attendant vacuomes, both the former contributing to a single acrosome. In fig. 6 the large cell formed from three nuclei and cytoplasms has a single group of abnormal-looking vacuoles (V).

We consider that X-radiation has no direct effect on the vacuolar system of the cells examined.

The "Giant Spermatids."

Giant spermatids may be of two classes. True spermatids, which have coalesced together to form large cells, such as depicted in Plate 19, fig. 6, and false spermatids, which are really either primary or secondary spermatocytes in which the nucleus is spermatid in form, and the secretion of the acrosome has been effected.

We have already referred to the peculiar second spermatocytes, which are perfectly normal cytological specimens except for their size, as shown in Plate 19, fig. 4. Such examples are photographed in Plate 20, figs. 10 and 12, and the details of acrosome formation are shown in the subjoined photo in fig. 12 *bis*. These cells are of much interest to us because they show that normal acrosome formation may occur in a cell which has lost the power of mitosis.

Other giant spermatids may be formed by a direct metamorphosis of primary spermatocytes which have concluded the final stages of the maturation pro-phases. Many such cells (Plate 18, fig. 2, N, 1st SPY.) are unable to enter mitosis, and consequently, while alive, become pseudo-spermatids, as shown in Plate 18, fig. 2, AN, 1st SPY. These are cells, which, as Strangeways believed for his tissue-cultured cells, were struck at some critical period before they got to the verge of karyokinesis. Acrosome formation in them is rare, but may occur even while their chromosomes are appearing, as shown in the microphoto in Plate 20, fig. 11, G.

The third form of giant spermatid, the true type, is interesting, and may be

really very large, as shown in Plate 19, fig. 6. In such cases we may get normal orientation towards spermatid tube lumen, normal giant acrosome formation, normal outgrowth of multiple centrosomes and tails, normal middle pieces, but a sort of "softness" in the nuclei and cell walls, which causes coalescence. Such cells never go far, and soon become collapsed and pycnotic.

X-rays and Sperm Motility.

There are numbers of researches which have shown that X-radiated sperms are injured as vehicles of heredity and of fertilisation. We have irradiated sperm emulsions for varying lengths of time and were never able to show that X-rays either increased or decreased motility. The case was different in spermatozoa taken from live irradiated guinea-pigs. Here for up to a fortnight after even very large doses ($8\frac{1}{2}$ times the human erythema dose), we were sure that sperms from the epididymis of X-rayed cavy were not only more active but remained alive longer than those from the control. At longer periods after irradiation complete immotility of spermatozoa is produced.

We have noticed the curious fact that such dead sperms appear normal in the vast majority of cases, and we consider that while the sperm is still inside the cytoplasm remnant and fixed on the wall, it develops normally, but perishes when it passes into the stream of effete material within the tubule lumen. The at first increased motility, and later the immotility of the sperm, are hard to explain. It might perhaps be due to a testicular toxæmia produced by the detritus of dead cells choking the lumina of the tubules.

Discussion.

One of the most curious facts which we have been able to show is that inability to undergo karyokinesis does not also mean that other important cell functions are no longer possible. By a study of gently irradiated cavy testis it has been shown that acrosome formation may take place quite normally in a cell which, so far as ability to mitose is concerned, is definitely injured. It may be considered that it has been demonstrated as likely that the inability to undergo mitosis is not connected with disfunction of the Golgi-idiozome complex. Caution is needed here, because it should be remembered that the proacrosomic material has been formed within the Golgi apparatus during growth of the spermatocyte, and the function of acrosome formation simply consists in the running together of these proacrosomic granules within the Golgi apparatus, and their deposition on the nucleus.

Some of the older workers considered that the injury caused by X-rays was

due to breaking down of the cell lecithin. Now the only lipid bodies (using the word "lipoid" in a wide sense) in the cells under consideration exist in the Golgi cortex and the mitochondria. As will be seen in Plate 20, fig. 9, and in fig. 14, the only bodies blackened by the osmic in the presence of chromium salts (Kolatchev method) are the Golgi apparatus, and the mitochondria more rarely. Most cells show only the Golgi body blackened. There may, of course, be lipid bodies in the ground protoplasm, but the osmic methods do not show these.

Now at normal mitosis the Golgi body breaks up and becomes scattered in the form of fine granules in the cytoplasm. If we assumed that this process was in some way essential to mitosis, and if further we considered that X-rays "hardened" the lipid cortex of the Golgi apparatus, and prevented its breaking up, then the experiences of Strangeways and Hopwood would be explained.

But does such a hypothesis fit in with broader facts of karyokinesis in embryogeny? In *Ascaris* eggs, where delay in development and abnormal development can be caused by X-radiation, the Golgi bodies are known to be scattered throughout the cytoplasm as in other eggs, and there is at present little evidence that such lipid bodies take part in mitosis. The adherence of Golgi dictyosomes to the asters in many male germ cell mitosis, and in mitosis of vegetative cells, might be explicable merely in the view that distribution of the Golgi elements to the daughter cells is the reason for the presence of such bodies in the amphiaster. Nevertheless there is now a large body of evidence that the Golgi dictyosomes lose staining power at the prophases, and only regain it at telophase.

This matter was discussed by the senior author some years ago (1918)—"It is evident, therefore, that during prophases of the maturation kinesis, the (Golgi) batonnettes as well as the mitochondria lose much of their affinity for stains, and cannot be demonstrated without recourse to specially heavy chromatisation of the material, and long mordanting and staining.

"These facts show : . . . that the batonnettes undergo some chemical change during the prophases of division, and do not resume their normal reactions to fixatives and stains until after the formation of the spermatid nucleus.

". . . That the Golgi rods being demonstrable by special methods during maturation divisions, do not take direct part in the formation of the amphiaster, though it is possible that some substance in them might have been withdrawn to give rise to part of the amphiaster . . . and is best known in the case

of the batonettes or Golgi dictyosomes of mollusca where loss of staining at the prophase is easily demonstrated" (10).

We might put forward the hypothesis that X-radiation caused alteration in the supposed (lecithin ?) lipid cortex of the archoplasm (sphere), that the cell could recover from such X-radiation, but that when the X-radiation synchronised with the prophase, the recovery became impossible, and essential elements of mitosis being therefore disturbed, abnormal karyokinesis followed by cell death occurred. The experiences of Strangeways and his associates, as to X-rays causing stoppage in mitosis of cells in the prophase, and as to abnormal mitosis occurring in such cells which spilled over into mitosis despite the X-radiation, would be explained. We offer this hypothesis as a possible line which brings into relation the scattered biochemical work on X-radiated lecithin, and the experiences of cytologists.

It will be clear that we are inclined to consider that the acrosome of the second spermatocyte does not appear because the cell is unable to undergo mitosis, and because the proacrosomic granules inevitably carry out their own function, but we consider that the cell is unable to undergo mitosis because the presumably injured Golgi apparatus is unable to break up and take its part in the prophases of mitosis.

R. N. Mukerji has produced some remarkable evidence in the case of *Lepisma* spermatogenesis. Here X-radiation not only causes lag in spermateleosis, but induces the spermatocyte Golgi discs each to secrete acrosome substance. These Golgi discs are now well known in insect spermatogenesis. They float in the spermatocyte cytoplasm, and are carried into the four daughter spermatids, where in *Lepisma* they vary in number from one to four. Acrosome secretion from them only takes place normally in the spermatid, and only then at one period, but Mukerji has experimentally brought about this secretion while the discs float in the spermatocyte cytoplasm, in many cases away from the nucleus.

How are we to interpret such a result? Is it a last despairing effort of a cell injured beyond repair, or is it a specific hit of the X-rays on these discs? If we examine the prophase stages of the young spermatocyte nuclei, we cannot find any abnormalities in the chromatin. Moreover the riper spermatocyte nuclei look normal.

Turning to the idea that the centrosome may be the injured cell constituent, we are able to show that very strong doses of X-rays neither prevent proper orientation of the spermatid in relationship to the lumen, nor the proper outgrowth of the flagellum from the head centrosome. The centrosome is

presumably a proteid body, and is not a granulated artefact produced by fixation, because it can be seen *intra vitam* in certain favourable cells. The idea that the centrosome is hit by X-rays, and so that abnormalities of mitosis are produced, is an obvious and attractive hypothesis. Yet if the X-ray alters the centrosome, how is it that the spermatid tail grows out, that the centrosome divides normally, and that normal motility is preserved? This seems an insuperable difficulty to the centrosome hypothesis.

Strangeways and Hopwood were against the idea that mild doses of X-rays stimulated. The divorce between the laboratory workers and the clinicians is nowhere so marked as in this case, because we know that some gynecologists have been using X-rays as a supposed stimulant in cases of human sterility. Surely all recent laboratory work, such as that of Brambell and Parkes (5), shows that the ovary is extremely sensitive to X-radiation, and sterility is quickly produced?

We know that a large proportion of mammalian oocytes never reach maturity, atresia being common in any ovary we may examine. The large unwieldy oocyte normally is prone to become overbalanced and necrotic. Such cells are most radio-sensitive, and the addition of the destructive elements from the X-ray tube to the ever-present danger of atresia is not likely to produce beneficial effects on the patient.

Finally we must acknowledge that the subject of the biological effects of X-radiation is one of great difficulty and obscurity. The experiences of Muller on X-radiated *Drosophila*, and of Bardeen on the effects of X-radiating sperm suspensions, seem to show that the effect is one which also concerns the nuclei and chromosomes. Possibly, as other workers have pointed out, no one single hypothesis can cover the multiplicity of facts various observers have collected with regard to the effect of X-rays on many types of cells.

In attempting to put forward a new hypothesis of the effect of X-radiation at the prophase of mitosis, we realise acutely our ignorance of the various physiological factors concerned in this process.

Summary.

1. It is suggested that the specific effect of X-radiation on cell mitosis is due to the temporary breaking down of certain lipid substances necessary for mitosis.
2. This lipid substance is supposed to be located in the cortex of the sphere (Golgi apparatus).
3. In a number of experiments it has been found that after X-radiation, the

Golgi lipid cortex becomes flocculent and granular, instead of smooth. This effect was not obtained in all experiments.

4. It is suggested that the explanation of the experience of Strangeways and Hopwood, that the most radio-sensitive period is just before prophase, is that the cell at this period is in a state in which recovery from the effects of broken down lipoids is impossible, so that an abnormal mitosis leading to death is the result.

5. The view that some substance, probably of a lipid nature, is drawn from the Golgi bodies at prophase, was put forward some years ago by the writer as a result of a study of molluscan dictyokinesis.

6. Assuming that X-radiation alters the lipid cortex of the Golgi apparatus, it may be suggested that cells entering mitosis might be killed by the inability of the centrosome to pass out of the idiozome preparatory to amphiaster formation. This hypothesis is now being tested with lymph tissue.

7. Inability to undergo mitosis, such as the first or second maturation division, in X-radiated testis, does not necessarily lead immediately to cell death, because such spermatocytes, both primary and secondary, may proceed to acrosome formation and the other preliminary stages of spermatoleosis.

8. Some interesting abnormal cells are described.

9. No evidence that the centrosome is radio-sensitive was obtained.

10. Likewise for the vacuolar system. The post-nuclear bodies are sometimes abnormal in X-radiated testes.

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- (26) Strangeways, T. S. P., and F. L. Hopwood, "The Effects of X-rays upon Mitotic Cell Division in Tissue Culture *in vitro*," 'Roy. Soc. Proc.,' B, vol. 100 (1926).
- (27) Wakelin, Barrat and Arnold, 'Archiv für Zellforschung,' vol. 7, p. 242 (1911); see Colwell and Russ.

DESCRIPTION OF PLATES.

Lettering.

A.,	acrosome.	D. SGN.,	dead spermatogonium.
A. 1,	inner part of acrosome.	G.,	Golgi apparatus.
An., 1st SPY.,	abnormal primary spermatocyte.	G. 1 and 2.	two parts of Golgi apparatus.
An. STD.,	abnormal spermatid.	G. SGN.,	spermatogonial Golgi apparatus.
AO.,	outer part of acrosome.	G., 2nd SPY.,	secondary spermatocyte Golgi apparatus.
C.,	centrosome.		
CB.,	chromatoid body.		

GX.,	Lagging Golgi apparatus of spermatid.	RSH.,	ripening sperm head.
INT. T.,	interstitial tissue.	SER.,	Sertoli cell.
M.,	normal mitosis.	SER. G.,	Sertoli cell Golgi apparatus.
N.,	nucleus.	SGN.,	spermatogonium.
NS.,	necrotic spermatocyte.	1st SPY.,	primary spermatocyte.
N. SGN.,	necrotic spermatogonium.	2nd SPY.,	secondary spermatocyte.
N. SPY.,	necrotic spermatocyte.	STD.,	spermatid.
N., 1st SPY.,	normal first spermatocyte.	STD. G.,	spermatid Golgi apparatus.
PNG.,	Post-nuclear granules.	V.E.B.,	Von Ebner's granules.
RS.,	ripe sperm.	Y.SP.,	young spermatocyte.
		X.,	perinuclear canal of spermatid.

PLATE 17. (Kolatchev, Camera Lucida.)

FIG. 1.—Normal spermatogenesis.

PLATE 18.

FIG. 2.—X-rayed spermatogenesis.

PLATE 19. (All Kolatchev, drawn with C.L.)

FIGS. 3 and 8.—Normal stages in metamorphosis of spermatid into spermatozoon.

FIGS. 4 and 5.—Secondary spermatocytes with acrosome formation.

FIGS. 6 and 7.—Giant spermatids formed by union of two or more normal-sized spermatids.

PLATE 20. (Photos taken with Leitz, "Mikam" Camera. FIG. 9, normal; the rest X-rayed.)

FIG. 9.—Normal testis showing Golgi bodies.

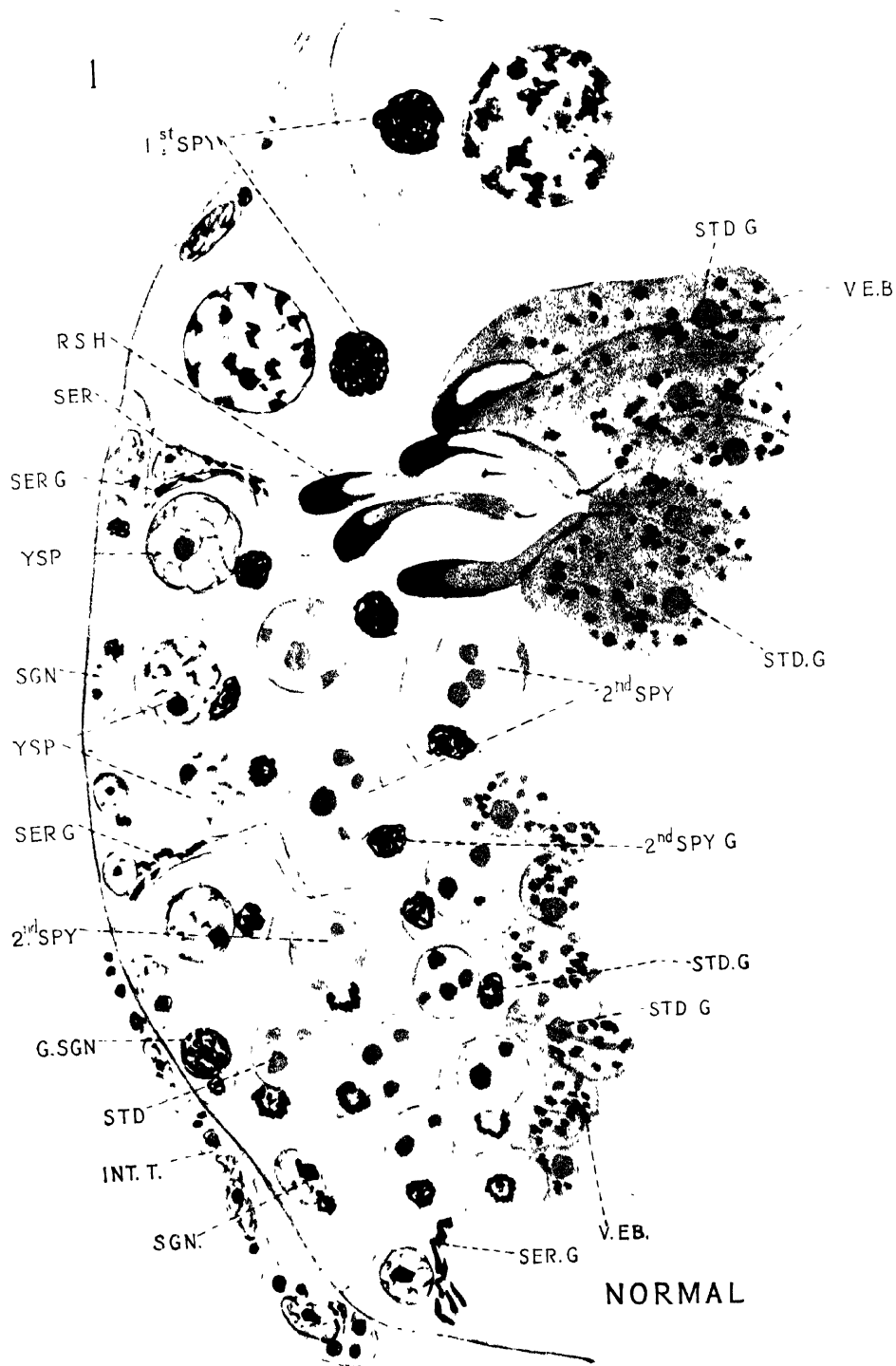
FIG. 10.—X-rayed testis showing undivided second spermatocytes.

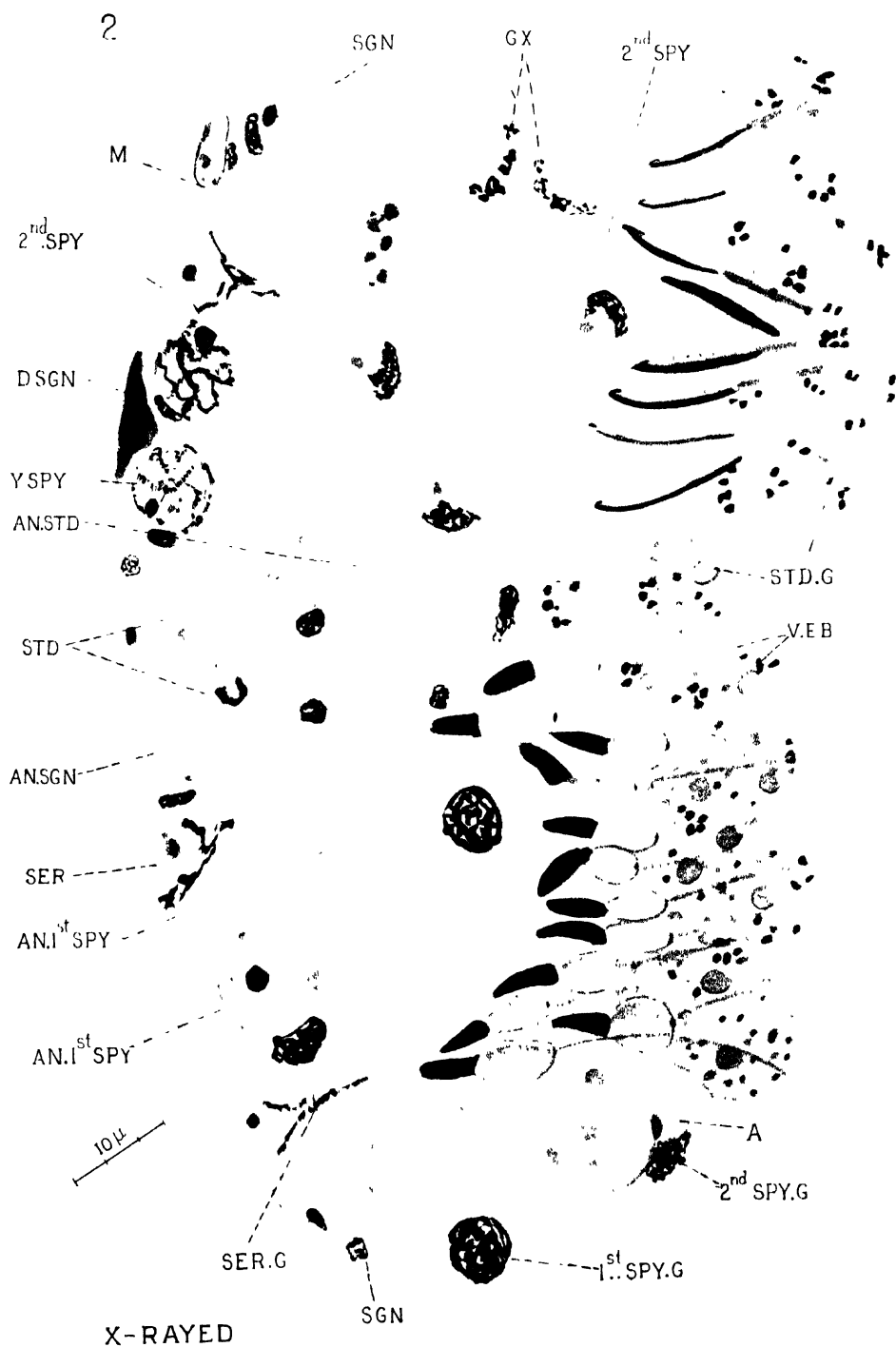
FIG. 11.—Acrosome formation in nearly adult spermatocyte.

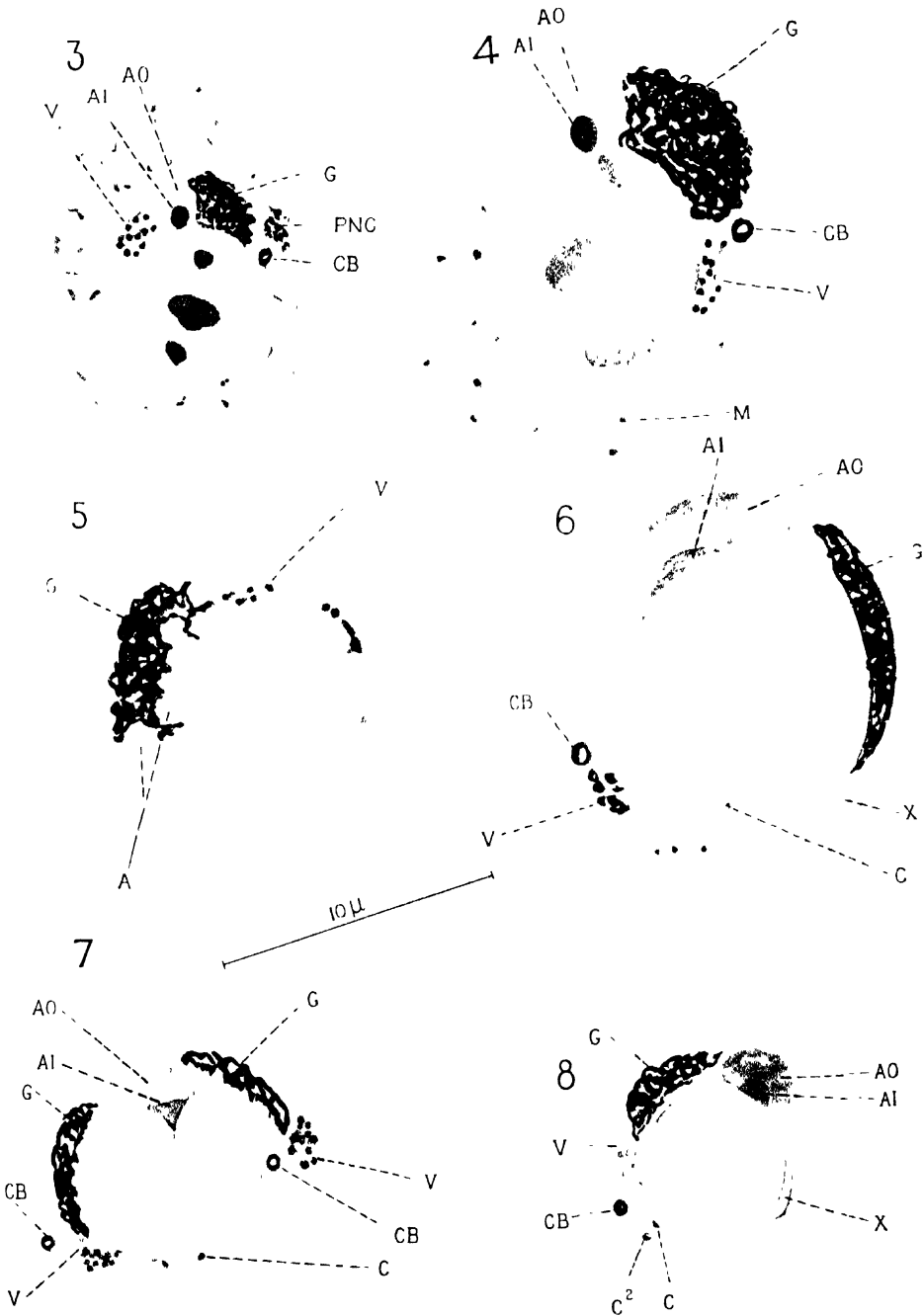
FIGS. 12 and 12 bis.—Secondary spermatocyte with acrosome formation.

FIG. 13.—Severely X-rayed testis into apparently normal spermatogonia. Four days after last dose.

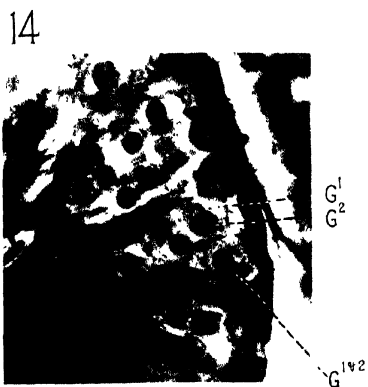
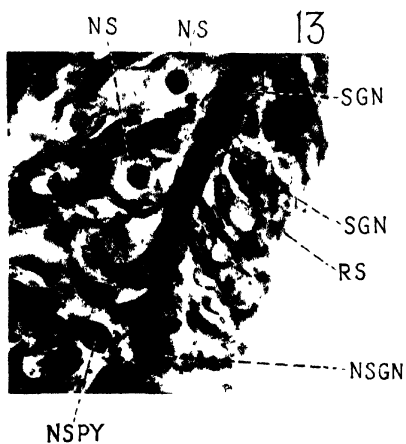
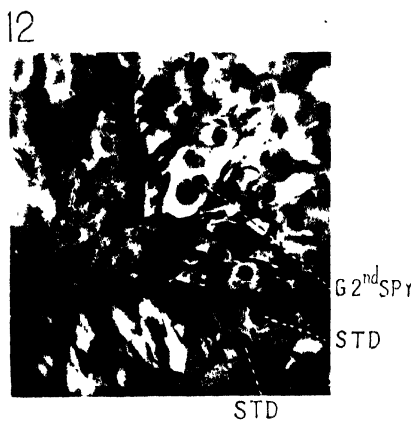
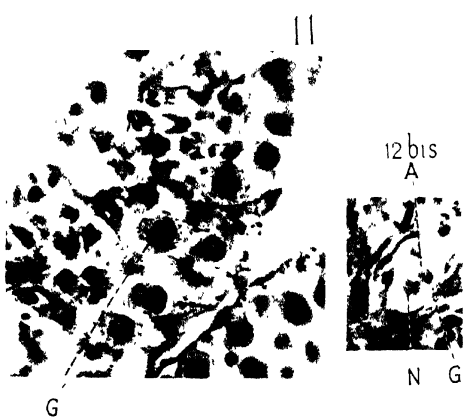
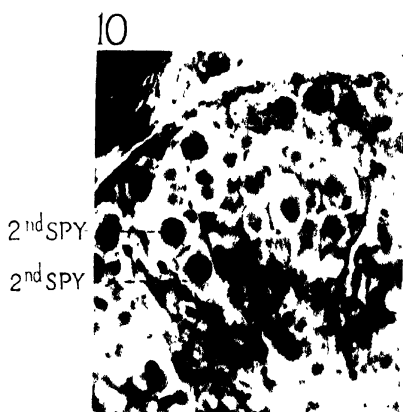
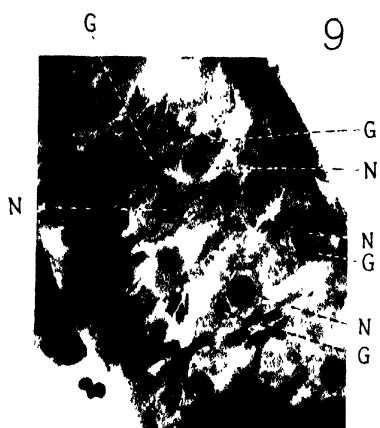
FIG. 14.—Secondary spermatocyte divisions showing lag in Golgi apparatus (G. 1 and 2).







FIGS 3 & 8 NORMAL



*The Histological Features of Striped Muscle in Relation to its
Functional Activity.*

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(From the Physiological Laboratory, Oxford.)

[PLATES 21, 22.]

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1. *Introduction.*

Analysis of the reflex behaviour of striped muscles involves data drawn from their histological structure, and the present observations deal with the differences in microscopical and macroscopical structure of muscles of different contractile properties. Stefano Lorenzini (55) mentioned the striking difference in colour between certain muscles of the limb in the rabbit as long ago as 1678. The classic investigations of Ranvier (63, 64, 65, 66, 67, 68), however, revealed most of the present knowledge of red and pale muscle. He showed that this redness was associated with slowness of contraction, and with the genesis of tetanus at lower rates of stimulus. Moreover, these functional differences

were shown to be associated with a difference in histological structure, a relatively larger amount of granular sarcoplasm and more distinct longitudinal striation in the case of red muscle. Later studies (1, 57, 43, 72, 49) revealed the occurrence of these granular and longitudinally striated fibres in muscles in many species, and also that these fibres were not always associated with redness of pigmentation (57).

The histology of muscle in the higher mammalian forms has shown that both granular "sarcoplasmic" and clear "aplasmic" fibres occur side by side in the same muscle, and these two types of fibre have been homologised with the red and pale muscles of the rabbit (29, 30, 43, 73, 42).

Ranvier (66), and later, Hay (35), and Lee, Guenther and Meleney (50) found red muscle more difficult to fatigue than pale muscle. A number of writers (49, 43, 70) have pointed out that the varying distribution of red sarcoplasmic muscle in wild fowl would seem to indicate that muscles used for prolonged and sustained effort are of this structure. Grützner (31, 32) arguing from the fact, confirmed by Basler (4), that the more slowly contracting fibres in mixed muscles tend, by reason of their slower relaxation, to obliterate the troughs between the successive peaks of the twitches of the more rapid fibres in a partially fused motor tetanus, supposed that all tonic functions were served by the one granular set and clonic by the other. Later Bottazzi, from his studies (9, 10) on the tonus of the tortoise auricle, put forward the theory of the tonic function of sarcoplasm, and considered that it was the sarcoplasm in red muscle which caused its prolonged contraction.

Attention to the subject was especially renewed when Hunter (37) and Hunter and Latham (38) pointed out that of the two types of nerve ending illustrated by Tschiriew (77) and Kulschitsky (48) in snake muscle the medullated fibre innervated thick muscle fibre, and the non-medullated thin, and suggested a plastic tonic function subserved by a slender type of fibre, and sought to identify these slender fibres with granular red fibres in mixed muscles. It is only in the ray, however, that Ranvier found granular slow fibres smaller in cross-section, compared with clear rapid fibres (63). In the rabbit he found the typical fibre of M. "semitendinosus" [red] of much the same diameter as that of the pale "vastus internus" (pale "adductor magnus") fibre. Meyer (57) finds the red fibre of "semitendinosus" in the rabbit over twice as great in cross section as the pale. Paukul (61) later found no very marked difference in thickness in the rabbit, both pale rapid and red slow muscles containing fibres of 70-120 μ . Arloing and Lavocat (1) confirmed Ranvier's figures for the ray and other fish, but found that in the dark leg muscles of birds the granu-

lar fibres were more voluminous than in the pale wing muscle. In higher animals they found light and dark fibres of equal size. Ewald (18), upon careful analysis, finds the clear fibres of the breast muscle of the pigeon to have 2.5 to 3 times the sectional area of the dark fibres.

The present position, then, indicates that the light and dark fibres seen in the muscles of vertebrates probably represent, in general, rapid and slow types of fibre; and these two types in the rabbit are found grouped as the pale and red muscles. As far as fibre diameter is concerned, the evidence of its affecting the type of contraction is more conflicting.

Fulton and Cobb (25, p. 142) found that in the cat, shown by Fischer (19) to possess a slow red soleus muscle, as does the rabbit, the soleus responds to break-shock stimulation by a slow twitch, which presents all the characteristics of the twitch of mammalian pale muscle in slower sequence. Fulton (25) also points out that the red element in a mixed muscle would cause a delay in relaxation of the motor twitch.

2. *Method.*

The muscles to be investigated have been isolated by section of the nerve branches to all others in the limb, except the psoas and short hip muscles, which were immobilised by tendon section. This preparation was made under deep anaesthesia, the animal then being immediately decerebrated under the same anaesthesia, and an interval (generally three hours and over) allowed for the effects of anaesthesia to pass off.

Fixation was as usual by metal drills, two stout drills for each bone concerned, clamped to the steel upright of the myograph table. The muscle pulled downward upon the myograph, which was of the Sherrington torsion-wire type. Double myographs were used frequently, either two wires end-to-end with their free ends together, and moving in the same direction, or else two wires side by side with movement of the recording limbs away from each other. The fixation of the myograph has been of the Sherrington girder type throughout.

The image of the myograph has been combined, in the same optical system, with the shadow of the string of the string-galvanometer and the two recorded upon the same plate, as for earlier work (15, 16).

For recording contractions of the external ocular muscles the skull was fixed by two brass clamps, at right angles to each other, one clamping the parietal portion of the cranium of the opposite side and the other the occipital portion, the frontal and parietal portions of the cranium and the roof of the

orbit being removed on the side of the recording muscles. The myograph was of the torsion-wire pattern with mirror magnification as described by Sherrington (14).

3. *The General Characteristics of the Motor Response of Mammalian Muscle.*

The isometric motor twitch of the extensors of the ankle of the cat is illustrated in text-fig. 1, A. The curve of tension rises abruptly and passes

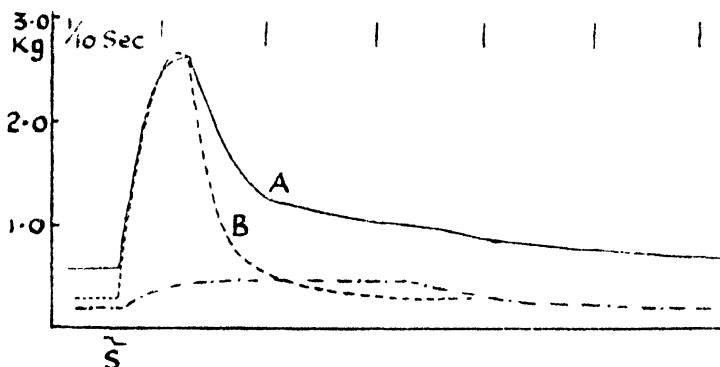


FIG. 1—A. Tension curve of twitch contraction of the gastrocnemius-soleus ankle extensor from excitation of the peripheral cut sciatic nerve by break shock at the fall of the signal (s). B. Superimposed tension curve of twitch of gastrocnemius alone after freeing the tendon of soleus from the tendo Achillis. The distance from the femoral condyle to the attachment to the os calcis was kept the same, although the tension had lessened. Same stimulus as in A. The dot-and-dash curve is the twitch of the soleus at the same length as in A in response to a break shock to its muscular nerve branch. All figures read from left to right. Time is indicated with each by vertical strokes traced from the shadows of the time marker spokes on the original photographic record (Rayleigh wheel). All text-figures traced from original plates.

gradually into a short plateau, ending in a sharp fall commencing abruptly 73.6 σ after the onset of the action current (which is not here shown), thereafter falling gently, especially in the latter part of relaxation. Occasionally a definite second plateau with a late second angle forms a definite hump to the late relaxation.

Sherrington (74) demonstrated an angle between the plateau and relaxation in the isometric twitch of mammalian muscle (tibialis anticus), and in other muscles examined by Liddell and Sherrington (52) a similar angle and a smooth relaxation were recorded. As Fulton (21, 25) showed, the normal isometric twitch of frog muscle is a similar rapid rise in tension gradually turning into a level or declining "plateau," which reaches a sharp "angle" 35-80 σ after

the onset of the action current, thereafter declining, at first rapidly and later more slowly, to reach the resting tension in a smooth concave curve. Fulton, in a series of careful analyses, in frog muscles with intact blood supply (20, 21, 22, 23, 24) revealed the effect of factors of temperature, initial tension, shortening, and summation, on the duration of the twitch, measured from the onset of the action current of the "angle."

In the cat the extensors of the ankle are three. *M. Gastrocnemius* consists of two heads, an outer and inner, each taking origin from the posterior surface of the corresponding femoral condyle and inclining toward its fellow. The aponeurosis of insertion of each blends with that of the other to form part of the tendo Achillis which is in its turn inserted into the os calcis. Deep to the outer head of *M. Gastrocnemius* is found a smaller muscle, much redder in colour, arising from the posterior surface of the tibia and having its tendon of insertion included in the outer side of the tendo Achillis. This is *M. Soleus*. If the deep surface of the external head of *M. Gastrocnemius* be examined it will be found to show a portion deeper in colour than the remainder of the external head, although not so red as *M. Soleus*. This muscle of intermediate colour takes origin from the aponeurosis of the external head and is inserted into a separate narrow tendon which passes medially to the portion of tendo Achillis derived from *M. Gastrocnemius*, and lower down comes to lie superficial to the latter. In this position it passes superficial to the insertion of the tendo Achillis and has no attachment to the os calcis but passes over a bursa to the sole of the foot, where it becomes the origin of a small intrinsic muscle of the foot. This extensor of intermediate colour is the *M. Plantaris*, which, besides causing extension at the ankle, also causes extension of the toes.

The twitch in text-fig. 1 is a response from combined *M. Gastrocnemius* and *M. Soleus* evoked by a break shock applied to the peripheral portion of the cut sciatic nerve. On account of the difficulty of fixing its tendon to the others *M. Plantaris* was always stripped back and took no part in the mechanical twitch.

If the insertion of *M. Soleus* be followed down into the tendo Achillis and detached from the calcaneum with a piece of bone, and the twitch response of *M. Gastrocnemius* be recorded alone, at the same original length, it presents the appearance of a simple twitch (text-fig. 1, B) of tension similar to text-fig. 1, A, although the initial tension is now lower. The tension record shows all the characteristics of the twitch of *M. Tibialis Anticus* in the cat described by Sherrington and of the twitch of frog gastrocnemius described by Fulton. The curve is now a simple one, with one plateau and "angle," and simple relaxation. The angle occurs earlier (at 68.2σ after the action current) than in the

curve A, but it is seen that the initial tension is lower owing to the removal of soleus and, as shown by Fulton (23) for frog muscle, and as will be described later for mammalian muscle, the higher the initial tension the longer the duration. The relaxation falls more rapidly in both its early and late periods and reaches resting tension very much earlier.

If now the M. Soleus be attached to the myograph and a similar break shock applied to the popliteal nerve where it leaves the under surface of the external head of gastrocnemius a low twitch curve results (text-fig. 1, C). The angle in this case occurs 260σ after the onset of the action current and the relaxation is thereafter smooth. The relaxation A can be derived from the addition of curve B and curve C. The relaxation of the combined muscle is the algebraical sum of the two simple curves, the "hump" or "*nase*" in this case being the expression of the remaining tension of the slow soleus which had not reached its angle. This confirms the finding of Fulton (25). In other muscles the "hump" or "*nase*" is less clear. Thus in the whole quadriceps a slow relaxation is all that is seen of a slow component which is found in crureus. In triceps brachii a similar slow twitch is found in the medial short head. Both these slow muscles are of a deep red colour similar in shade to that of soleus. In each of these cases the "*nase*" in mammalian muscle is due to the slow contraction of a deeply pigmented portion of the muscle, shorter and smaller than the rest.

Over a large series of animals the duration of the twitch of gastrocnemius, when separated from soleus, measured from the onset of the action current to the "angle," varied between 40σ and 140σ , while that of soleus varied between 120σ and 440σ . When measured concurrently, in response to the same break shock stimulus to the popliteal nerve in the thigh, the latent periods of the action currents of gastrocnemius and soleus showed a difference of the order described by Kohlrausch (44A). The onset of the mechanical response in soleus is also slower than that of gastrocnemius—in the double concurrent string and myograph record, from which the above electrical latencies were measured, the latency of the mechanical response of soleus was always longer than that of gastrocnemius (e.g., $10\cdot0\sigma$ compared with $6\cdot5\sigma$). In this the findings of most of the earlier workers are confirmed. The longer latency of soleus in the cat was not found by Fischer (19), but, as will be seen later, his curves from soleus show undoubted evidence of an early rapid component, not belonging to this muscle.

In the same animal the tension developed by the soleus twitch is always much smaller than that developed by gastrocnemius. Under fairly comparable

conditions, *i.e.*, comparable initial tension and shortening, a twitch of the internal head of gastrocnemius will develop from three to six times the plateau tension of a maximal twitch of soleus. The nerve threshold for excitation of the rapidly contracting gastrocnemius is in general lower than that for the slow soleus, as noted by Fulton (25), though occasionally the reverse (for both faradic stimulus and condenser discharge) occurs and was commonly found in the slow element of supraspinatus (16).

The motor tetanus produced by a repetition of stimuli to the motor nerve at a rapid rate shows (text-fig. 2) differences in soleus and gastrocnemius. The ascent is slower and the plateau "after-action," or interval between the last stimulus and the angle of commencing relaxation, much more prolonged, and the fusion of the tetanus occurs with a much slower rate, in soleus than in gastrocnemius: features described for the red and pale muscles of the rabbit by Ranvier and others, and for soleus and gastrocnemius in the cat and rat by Fischer (19). Owing to the difference in size of the two muscles in the cat, the comparison of maximal tension developed does not convey much information of muscle fibre difference, but it is certain that under isometric conditions the soleus develops a much smaller maximum tension (1.3 kg. in the above example) than one head of gastrocnemius (6.0 kg.), and never less as was stated by Fischer.

A rough approximation of comparison in tension development can be made by weight. For instance a soleus developed 1300 grams maximal tension and the muscle, less tendon, weighed 1.41 grams (weight of cat 1.3 kilo). The associated internal head of gastrocnemius developed just over 6 kilograms in maximal tetanus and weighed 2.28 grams. This means that soleus developed 922 grams tension per gram weight, while gastrocnemius developed 2631.5 grams tension per gram weight. The ratio of fibre tension in the one to fibre tension in the other cannot be found exactly with present apparatus, but allowing for the greater fibre length in soleus ($\frac{1.45 \text{ cm.}}{1.05 \text{ cm.}}$ in this case) the tension development per unit fibre in soleus must be some 48 per cent. of that of gastrocnemius or slightly more, say 50 per cent., as the fibres in soleus are larger.

The ratio of the tension developed in a maximal motor twitch to that developed by a maximal motor tetanus is much smaller in soleus (1 : 4) than in gastrocnemius (1 : 2). Grützner (32) found the ratio 1 : 3 or 4 for gastrocnemius and 1 : 10 for soleus in the rabbit (isotonic records, where shortening is an additional factor). The difference in ratio between the two muscles is sufficient to indicate a difference in the quantity of surface or material available for activa-

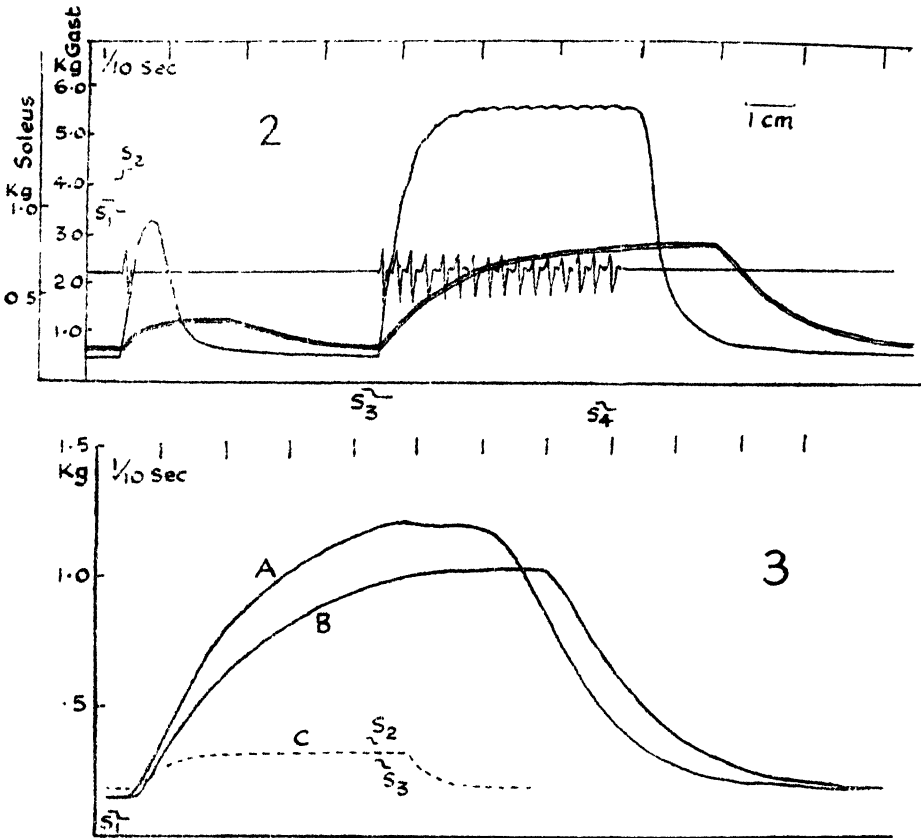


FIG. 2.—M. Soleus and Gastrocnemius, motor twitch followed by a motor tetanus. Stimulus to sciatic nerve at rate of 50 break shocks a second, of which one shock is allowed between signal s_1 and signal s_2 , and 16 between s_2 and s_4 . The action currents are from leads in soleus. Double myograph with pull in same direction. The pointer for soleus records a double curve by means of a fine hair attached next to the steel point.

FIG. 3.—M. Soleus. Motor tetani in response to stimulation at 50 a second. A. Stimulus to sciatic nerve, between signals s_1 and s_2 . B. The same, after section of a small strip of fascia attached to the medial border of soleus. The delayed relaxation relative to A is due in part to the raising of the initial tension which fell when the fascial strip was sectioned, and partly to the later arrival of the last stimulus of the series (between s_1 and s_3). C. The same muscle, before section of the medial fascial sheet, response to less strong stimulus applied to the sciatic. No action currents in soleus, contraction (apparently maximal) in gastrocnemius.

tion by one wave of excitation, relative to the total potential surface or material. A differing rate of disappearance of a material used in contraction would also be an explanation. The slower rise of tension of the motor tetanus of soleus

is probably an expression of the same phenomenon, and is independent of the rate of stimulus.

The plateau "after-action" in a fully developed motor tetanus is in both muscles a little less than the duration of a motor twitch in the same preparation. This is characteristic of the after-action of a motor tetanus in frog gastrocnemius as described by Fulton (22), except that the mammalian muscle does not increase the after-action until the motor tetanus has been sustained a considerable period. This latter difference is probably due to the more rapid circulation in mammalian muscle.

In his study of summation in frog muscle Fulton (24) described the augmenting effect of one excitation upon the duration of a response to excitation within an interval of 40 σ later, and he found that the maximum augmentation of duration of the second response occurs at a moment very shortly after the arrival of the first stimulus. In gastrocnemius of the cat such augmentation of the duration of the second response is minimal, and indeed it often occurs that the second of two responses close together is actually shorter than that produced by the second stimulus alone.

Soleus, on the contrary, often shows well the augmentation of duration of a second twitch, occurring early (with an optimum slightly later than the least interval for the effect of the second impulse) after the first excitation, and obtainable as late as the angle of the first contraction. A short tetanus of this type of soleus shows tremendous enhancement of peripheral after-action, occurring with a maximum after the last of a series of five shocks at 50 a second, and being then greater than after two shocks or ten shocks at the same rate, and gradually lessening as the tetanic response reaches the plateau (illustrated in a recent paper (15)). This augmentation has been found after stimulation of the peripheral ends of the cut ventral roots supplying the muscle (*i.e.*, avoiding stimulation of the sympathetic), and is also found when the motor nerve is stimulated in continuity during inhibition of the centre. In this latter case the phenomenon has lately been used as a criterion of the rate of reflex discharge (15). Once the plateau of the motor tetanus is reached, the after-action behaves as for pale muscle, reaching the angle within the duration of the twitch response until fatigue again delays it.

Rapid muscles have been encountered in which the second response of two summated twitches is of longer contraction-duration than one alone, but these muscles do not show any great augmentation after a short motor tetanus. Similarly soleus may occasionally not show augmentation of the second response when summated, and may then show but slight augmentation of

contraction-duration after a short motor tetanus. The amount of augmentation of contraction-duration in the process of summation is in general greater, the greater the duration of the muscle. The muscle contraction-duration increases with fall of temperature, and with it the process of augmentation of the second and later responses.

4. *The Homogeneous Motor Response.*

If both soleus and gastrocnemius retain their muscular branches from the popliteal nerve, sectioned higher up, and records of a motor twitch of soleus in response to a break shock to the popliteal be made, it is found to be extremely difficult to avoid a slight early rise of tension, and fall in the plateau, due to the vibration or pull of gastrocnemius. This transmission of effect occurs though the tendons be freely separated. A small strip of fascia attached to the medial border of soleus is the cause of most transmission between the two, and once this is removed the twitch of soleus follows the simple slow curve which is obtained by stimulation of the small nerve to soleus alone.

This transmission of the mechanical contraction of gastrocnemius to soleus provided, however, a convenient criterion of effects caused by compounds of muscle fibres of different contraction-duration. It was found that a muscle giving the double twitch curve, a mixture of rapid and slow curves, would show evidence of the mixture even more clearly in the curve of a motor tetanus, where the ascent is a little steeper than in the pure slow curve and the peripheral after-action is markedly double. The after-action shows, in fact, the summed after-actions of each component, and the difference in height between the first and second angles is that of a tetanus of the pale fibres alone.

In preparations where the threshold of excitation of rapid muscle is lower than that of slow muscle, the rapid component in such a mixed tetanus can be obtained alone by using a minimal tetanic stimulus, and, if the difference in threshold be sufficiently great, it is possible to throw all the rapid fibres into tetanus with a strength of current which does not involve the more sluggish fibres. The extent to which this is true can be controlled easily by a double concurrent record. An instance of this is shown in text-fig. 3, where gastrocnemius was able to be stimulated maximally by electrodes to the popliteal nerve without involving soleus. Here, although a slightly earlier rise in tension was the only sign of transmission to the maximal motor twitch of soleus, a minimal twitch showed a small pale contraction of 70 grams tension. A maximal motor tetanus showed a double after-action (text-fig. 3, A), one fall in tension being only 20-30 σ after the last action current, and the delayed

angle, which was "blurred," is seen 130 σ later. A minimal stimulus produced a rise in tension of 0.25 kg. in soleus (text-fig. 3, C) which ended in an angle 50 σ after the cessation of the stimulus, and was not accompanied by action currents in the muscle. Gastrocnemius was the only muscle observed to move, and found to be in maximal tetanus. Section of a band of fascia along the medial border of soleus immediately resulted in the tension curve in soleus caused by a maximal stimulus to the popliteal nerve, changing to the form seen in text-fig. 3, B. The double curve of decline of soleus has vanished, leaving a simple curve with sharp "angle" (the after-action is here longer because the last wave of stimulus is later, and the initial passive tension has been slightly raised after section of the fascial band). This type of simple curve could be obtained earlier by stimulation of the nerve to soleus (and so not involving gastrocnemius).

Here a proportion of rapidly contracting fibre amounting to 0.25 kg. could be detected quite clearly from the preliminary fall in plateau after-action in a motor tetanus of 1.8 kgs. But whereas it is sometimes not possible to separate the two parts by varying the stimulus, it is always possible to see the most minute variation from the smooth after-action of the maximal tetanus curve, seen in text-fig. 3, A; and by this means it is estimated that a fibre content differing in duration by as little as 50 σ , and present in a proportion as little as 5 per cent., can be surely detected.

The complicating factor may arise from the under surface of gastrocnemius or of plantaris, and its duration varies with the tension put upon the fascia conveying it and not upon the tension in the muscle giving origin to it. A rapid component has been found complicating soleus and relaxing before gastrocnemius, although having origin from the latter as a small sheet of deep fascia. Fischer (19) found the latent period of soleus and gastrocnemius in the cat and rat to be the same, but, as his curves show the early rise typical of transmission, it is obvious that the latent period must have been that of gastrocnemius in both cases.

Soleus isolated in this way, and excited by a long tetanic stimulus which is submaximal, often shows a peripheral after-action which resembles that of pale muscle in its shortness, and this would appear to show that lower threshold fibres of short duration had been excited by the stimulus. There are two reasons to suppose, however, that such a short after-action is due to early defalcation of fibres of long duration. Firstly, if the same stimulus be stopped early, the after-action is then long, and secondly the action currents are always irregular when the short type of after-action occurs. If the action currents are regular

the after-action is long. Pratt (62) in his study of the responses of isolated fibres of frog muscle obtained an alternating rhythm of response when stimulating a single fibre tetanically at an intensity near threshold. The fibre responded regularly to every fourth stimulus. The irregular action current in such a soleus is evidence that here fibres are dropping away through raised threshold, only to appear later to maintain the tension for a short time. Such a submaximal tetanus becomes quite irregular if allowed to continue for more than 2 seconds. This phenomenon may appear to contradict the law of summation, but that the fibre is re-excited after an interval shows that this factor is still present, the defalcation being due to a raised threshold occurring after excitation, a phenomenon noted by Sosnowski (75) in the slow moving tissues of some invertebrates.

The rapid component in such motor contractions of soleus could always be abolished either by section of the fascial sheet connecting the muscle with the under surface of gastrocnemius and fully isolating the tendons, or by using only the nerve branch to soleus itself for excitation. The twitch and tetanus of soleus alone are always simple slow curves, and this held also in direct excitation of the muscle by pin electrodes. It is therefore possible to state that M. soleus in the cat is always composed of at least 95 per cent. of the slowly contracting type of fibre, although this method of analysis does not reveal minor differences in duration of an estimated order of $\pm 10\sigma$. In the routine examination of over 45 cats in this way, no exception to this statement has yet been encountered.

The motor twitch of the rapidly contracting muscle, gastrocnemius, has in most cases reached full relaxation at a time when soleus has not commenced to relax (text-figs. 1 and 2). It does not seem likely therefore that it can contain any type of fibre as slowly contracting as that of soleus, unless that fibre be in very small amount, or unless it be in some way prevented from causing any tension effect. It is less certain that variations in contraction-duration of as much as 25 per cent. do not occur, for the plateau of twitch and tetanus usually declines a little from an early peak, and the relaxation is so rapid as to obscure any late angles. The sharpness of the angle in the motor twitch, however, is some guarantee that little variation occurs.

5. The Relationship of the Structure of Muscle to the Duration of the Contraction Process.

In view of the variations in granulation and thickness of the constituent muscle fibres in gastrocnemius or soleus of the cat, which is just as variable in

this respect as other mammalia, it is to be expected that, if the conclusions of Grützner (30) and Basler (3) are true, the effect of maximal stimulation of the motor nerve of these muscles will be the sum of the different contraction effects of fibres of different types. Knoll (43) showed that clear fibres (free from granules, and therefore rapidly contracting, according to Ranvier) could be present in some proportion in muscle which contracted slowly; but there was then no adequate evidence to show that the mechanical effect of such a proportion was not exerting such a small effect that the myograph did not reveal its presence. Basler (3) was able to demonstrate the presence of two types of contracting element in frog sartorius, the one delayed in relaxation and fusing to form a tetanus at slow rates of stimulus, the other rapidly relaxing and needing a high rate of stimulus to fuse the twitches to form a tetanus. He sought to identify these two types with the thin and thick fibres in this muscle.

The myographic method described in the last section provides a criterion of the duration of the contraction process in a muscle such as soleus or gastrocnemius, and further shows that such duration must represent the summed individual mechanical effects of each muscle fibre comprising the muscle. These muscles are found to be formed of fibres of varying diameters when examined microscopically in transverse section, and of varying degrees of opacity when unstained.

It will be shown that the muscle fibres of the muscles of the cat vary in microscopical appearance according to the state of nutrition of the animal, and it will be further demonstrated that these changes have no effect on the contraction process. The thickness of a muscle fibre, and its granulation or opacity* are features which do not indicate any particular type of contraction process.

(a) The Method.

A soleus found myographically to be composed of at least 95 per cent. of fibres which contract with the same duration measured from action-current to angle, when fixed in formol (5 per cent. in saline), cut in frozen sections and examined unstained in water under a low power, shows a mixture of opaque and clear fibres. A similar varied

* It will be indicated later in this paper (*v. §§ 5, b, d*) that opacity (darkness in the unstained fibre), granular appearance (under high magnification), and the darkness with ordinary stains which gives the appearance of abundant sarcoplasm ("plasmic" compared with "aplastic") (42), are synonymous when referring to the type of fibre indicated by Ranvier and Grützner, and are each due to the same characteristic, namely a granulation composed of some substance containing combined fat. "Clear" and "opaque," "light" and "dark," "non-granular" and "granular" "aplastic" and "plasmic," therefore refer to the same two types of muscle fibre, the choice of adjectives depending on the means by which the difference is revealed.

appearance may be produced by fracture or knotting of the myofibrils within the sarcolemma by overstretching during fixation, as shown by Schaffer (78). That the fibre counts given later are complicated by this error is certainly not the case, as such a fracture or knot is easily recognisable, and the knots, though staining dark with gold or silver impregnation, stain very little differently from the rest of the fibre with the Sudan III of Ewald (18) which was used in this research. Also, such fracture is due to overstretching, and in the present experiments overstretching has been carefully avoided, and controlled by longitudinal sections.

It is obvious that for comparisons of fibre size it is absolutely necessary to adopt a standard method of fixation, and, after many trials, the following procedure was adopted as giving the most satisfactory results. A few minutes after the death of the animal a piece of the muscle to be examined was excised. The piece is narrow (1 cm.) parallel with the fibre but at least 3 cm. long in the long direction of the fibre (in a small cat a whole soleus for example). The piece is suspended by one extremity in formol saline (5 per cent. formol in 0.9 per cent. saline) and left for 24 hours at least, and for not more than three days. It is then washed in running water for 2 hours and placed in a gum sugar medium (made up by the formula given by Cole, without carbolic acid) for at least two days, and not more than five days. Frozen sections are then cut at 35μ and washed in distilled water. They were then examined with low illumination, for the unstained section is the most reliable index of the darkness of the fibres.

For critical work on the relative opacity of muscle fibres, and for photography, it is necessary to stain the sections. Osmic acid is, as will be explained later, too selective for the present purpose, and the alkaline Sudan III of Ewald (18) was used, giving in my hands results much superior to those given by Herxheimer's alkaline Scharlach R (Sudan IV). This solution must be freshly made up, as its staining properties rapidly weaken, even though excess of the Sudan III be present. It must be emphasised, however, that the true index is opacity in the unstained fibre, as used by the earlier workers (Knoll, Schaffer and others), and the pictures presently to be described rely on the stain only to emphasise the points seen in the unstained section. The stained section is mounted in water and photographed. The stain is not permanent and crystallises out within a short period, and to overcome this difficulty microphotographs were immediately made of a series of representative sections of each piece of material.

Two questions immediately arise: In how far does a cross-section portray the character of the whole of any particular fibre, and, is the contraction process of each muscle fibre represented in an isometric maximal motor contraction? In the cat the inner head of gastrocnemius is more simple in structure than the outer head and is found to consist of muscle substance between two fascial layers, one an aponeurosis attached at its upper end to the femur and fading into muscle substance at its lower end, the other an aponeurosis running into the tendo Achillis below and fading into the muscle substance above. The portions of these two aponeuroses related to muscle substance are in the form of sheets, the first diverging from the origin of the muscle, the second converging upon its insertion. Between these two sheets is the muscle substance, and if the muscle be cut through from one sheet to the other in a direction parallel to its direction of pull, and the cut edges of the muscle substance be examined, they are found to consist of numerous fasciculi running each from one aponeurosis to the other. These fasciculi run parallel to each other and obliquely from the aponeurosis of origin (which is superficial) downwards and forwards to the aponeurosis of insertion. By making many such sections it is simple to see that the internal head consists of hundreds of parallel fasciculi, and a further careful dissection of

the fresh muscle with a wet knife shows that every fasciculus runs from aponeurosis to aponeurosis. It was further found, by dissociating numerous fasciculi in 20 per cent. nitric acid for 24 hours, that in any particular fasciculus the fibres run from end to end of the fasciculus. In gastrocnemius, by thus dissociating fasciculi, no fibre was found which did not reach from aponeurosis to aponeurosis. All fibres, thin and thick alike, found their way from end to end of the fasciculus.

The external head is also built of parallel series of fasciculi, although here the aponeurosis of origin is subdivided into three ill-defined parts from which the fasciculi converge on the aponeurosis of insertion. The aponeurosis of origin also gives rise to the parallel fasciculi of plantaris. Soleus has two simple planes of tendon-aponeurosis as has the internal head of gastrocnemius, and all fasciculi are parallel. Dissociation of fasciculi of soleus only once revealed a fibre which did not reach the end of the fasciculus, although a large number of fasciculi were thus examined.

Thus each of these muscles consists of single fibrils lying parallel to one another in fasciculi which are in turn grouped in parallel series, and this arrangement can also be shown for each part of quadriceps (where the internal and external vasti possess some very long fibres as well as short ones). Rectus femoris, although possessing a long muscular belly, is arranged on the plan of the internal head of gastrocnemius, only in this case the two aponeurotic sheets are in turn folded about one another. The fasciculus length is short, although the apparent muscle length is long. So, too, for semitendinosus, while tibialis anticus is simply disposed, though the fibre lengths vary.

The structure of striped muscle therefore reveals that it is made up of thousands of parallel members, each exerting its longitudinal effect from one layer of aponeurosis which forms a sheet of origin, to another layer of aponeurosis which forms a sheet of insertion. When the origin and insertion are fixed each fibre member is therefore in the same relation to the aponeuroses. The fibres described by Lindhard (54) in the frog sartorius ending prematurely are found, in dissociations by the Sihler method, each to possess a fine tendinous band linking them with the tendon expansion: this tendinous band, like all tendon aponeurosis, is destroyed by nitric acid dissociation; and there is no reason to suppose that this type of fibre exerts its effect through other muscle fibre. The spiral muscle fibres described by Lindhard have not been seen in fresh muscle, or muscle dissociated by the Sihler method (*R. temporaria*). Certainly in the cat, rat, rabbit, dog, and monkey, for gastrocnemius, plantaris, soleus, tibialis anticus, semitendinosus (two sets of fasciculi linked end to end), and the components of quadriceps, the fibre systems are essentially simple in type, and each fibre receives equal representation in the direction of pull. This is considered to be the basis of muscle fibre arrangement in all striped muscle, although the complex arrangement in tenuissimus and sartorius in the cat await investigation.

In contraction, each muscle fibre attached to the same tendinous sheet of origin and sheet of insertion must therefore have the same mechanical advantage, provided that the origin and insertion are not approximated by the contraction. If shortening occurs it is conceivable that a fibre late in entering the contraction lacks the mechanical advantage of the greater initial length of the fibres first in activity. If the sets of fasciculi are not strictly parallel, as in a muscle with more than one head then, as others have described, the angle of pull affects the resultant mechanical advantage.

The internal head of gastrocnemius and soleus in the cat are two muscles which are ideal for recording contraction and for the purpose of histological examination. The fasciculi in each are simply disposed, their aponeuroses of origin and insertion are simple planes, and the attachment of each aponeurosis is discrete enough to form almost a point origin and point insertion. Isometric contraction should reveal the summed mechanical effects of all the fibres, and a series of sections across the muscle belly, at right angles to the direction of fasciculi, and spaced 0.5 cm. apart will present a transverse section of each fibre.

(b) *The Light and Dark Muscle Fibres and Fibre Thickness.*

If a cat of average size and weight be examined, soleus is found, for example, to give a slow twitch of 170 σ and not to show signs of any rapid element, as tested, by threshold, by twitch shape and by evenness of plateau after-action (*v.s.*, § 4, p. 381). A cross-section from this soleus is shown in fig. 1 (Plate 21). The computed proportion of rapidly contracting muscle fibres which could be present unrevealed in the myograph curves, and the proportion of histologically visible clear fibres, are perhaps not dissimilar. It is conceivable that tension exerted by the clear fibres is concealed in the massive response from dark fibre if these histological characteristics reveal respectively rapid and slow contraction characteristics as Grützner supposed. Gastrocnemius in this animal gave a maximum motor twitch of 56.5 σ , and the histological structure was that seen in fig. 2 (Plate 21). In this case gastrocnemius has fallen to 1/20 of its tension at an interval of 170 σ after its onset. Therefore only some 1/10 of its fibres could possibly have been physiologically similar to those of soleus, but it is seen in the microphotograph that at least some 30 per cent. of fibres are very dark. These microphotographs are chosen as fair samples of the general cross section appearance.

The dark or opaque fibres in both muscles, when examined in longitudinal section, show the Sudan staining material as minute droplets arranged along the striation (figs. 3 and 4, Plate 21). They evidently correspond to the J granule of Holmgren (36). Not only are they arranged transversely at the isotropic (clear) line, but they are also arranged longitudinally in rows of varying size and length. A given longitudinal row has its granules arranged opposite the clear lines but not opposite every clear line, and there is great irregularity in size in the granules. The striation is therefore apparently reversed in very dark muscle. A given longitudinal row does not extend through the whole muscle fibre but fades out after a varying distance, another

row making appearance, so as to keep up an average granulation through the length of the fibre. The longitudinal rows are evidently responsible for the "longitudinal striation" of earlier authors. The cross striation is, however, the same in both clear and granular fibre, the lines are the same distance apart (approximately $2\ \mu$) in each, and in granular fibres the striation is still well apparent between one longitudinal row of granules and another. It is quite clear that the rows of droplets are lying closely packed between bundles of myofibrils, as was shown by Retzius (69), Kölliker (15) and Holmgren (36).

The light and dark fibres can similarly be demonstrated in transverse and longitudinal section in snake muscle. Several varieties of grass snake have been examined and the results of staining with gold chloride and with Sudan III correlated, and it is certain that the small dark granular fibres in snake muscle stained with Sudan III (fig. 5, Plate 21) are those described by Tschiriew (77) and Kulschitsky (48) as innervated by endings "en grappes."

That the opacity of dark muscle was due to these "liposomes" was shown by the earlier authors, and that they are stainable by alkaline solutions of Sudan III and Sudan IV was shown by Bell (6). He considers that the droplets are triolein, but there is some probability that they are a breakdown product of some complex lipid, as Sudan III without sodium hydroxide stains them hardly at all, while osmic acid stains only the most coarse granules black. Further, it was found in these experiments that the opaque muscle in the rabbit and sheep stains but little, even with the alkaline Sudan, indicating possibly some difference in the hydrolysis of the complex.

Knoll and Hauer (44) first described the disappearance of the fat granules in pigeon muscle in starvation. It was demonstrated by Greene (27) that the granules in the lateral pink muscle and in the dark muscle of *Salmo* almost disappeared when the fish had completed the migration fast, and Bell (6) showed that the liposomes in the rat and ox disappeared in starvation.

Therefore the darkness of cat muscle should also be dependent upon nutrition. In order to test this a number of cats of varying states of nutrition were examined and the muscle duration, relative fibre size, and granule content (darkness, opacity) compared. These latter were measured by the Sudan method outlined above, in transverse sections $35\ \mu$ thick. The results for typical animals are expressed in the Table given below (p. 388).

The table is from selected preparations to show the salient features. Where the variation between size and size within a particular type has been small the average area is given, where the variation was large the limits are given. It is seen that fibre speed is not necessarily fast or slow with a fat or thin animal.

So too, fibre size does not necessarily follow nutrition—thus in cat 5 all fibres were small, but they are also small in cat 3 which is a normal cat. Cat 6, which was emaciated, is found to have quite large fibres in soleus although there is so little opacity. No feeding experiments were made and the animals were used in the condition in which they were received. Morpurgo (59) noted lessening in size of muscle fibres in starvation, as well as lack of fat, and it may be that it is only a matter of time of starvation, or type of diet in relative starvation, before the diameter lessens, while the Sudan staining substance disappears earlier. Morpurgo observed as much as 68 per cent. loss in diameter (pigeons), Bell (6) has observed 50 per cent. loss in cats and rats. In this connection it is perhaps not unnecessary to say that the fibre measurements in the table were not made in the vicinity of an aponeurosis, where all fibres are narrowing down before insertion. No attempt has been made to correlate the tension of contraction and the weight of the animal.

Occasionally, even in the most fat-laden muscle, as seen in soleus in fig. 11, Plate 22, an occasional minute pale fibre can be found and can be traced through the whole fasciculus. The dark fibres toward the border of gastrocnemius, seen often in flexor muscles also, are often much smaller than the rest. By analogy with the small tension produced by the eye muscles and their related small fibre diameter, it seems probable that these fine fibres also produce but small tension, and so are possibly capable of enlargement in the process of "training."

Soleus in all these animals was entirely and uniformly slow in contraction as judged by twitch and tetanus, and since in cats 5 and 6 it was composed of 97 per cent. and 99 per cent. clear fibres respectively, it follows that such clear fibre cannot be rapid fibre. Clear fibre cannot be responsible for the short duration of soleus in these cats relative to the duration in cat 4, because in cat 1 the contraction-duration of the soleus was shorter still, though it possessed more dark fibre.

In fig. 6, Plate 21, is shown a high-power view of one of the only dark fibres which could be found in gastrocnemius of cat 5 (emaciated), showing the clear state of the neighbouring fibre, and in fig. 7, Plate 21, is shown a low-power field from soleus also from cat 5. (See also fig. 19, Plate 22, from cat 6.) Gastrocnemius of cat 4 (fat) reached a negligible tension before soleus reached the angle. The section of this gastrocnemius is seen in low-power view in fig. 8, Plate 21, and in high-power view in fig. 9, Plate 21, and soleus in high- and low-power views is shown in figs. 10 and 11, Plates 21 and 22.

The differences between the opaque fibres is obvious from the sections of the

muscles. It will be noticed that the dark fibres in gastrocnemius in a very fat cat are considerably darker than those in soleus in the same animal, while there is a large content (48 per cent.) of absolutely clear fibre in gastrocnemius in an animal which is very fat. The wide variation in amount of dark fibre in the two animals 4 and 5 leaves only one conclusion, that the contraction process is not affected by the variation in number of dark fibres. The twitch and tetanus is the same for *direct* stimulation of the muscle by needle electrodes with strong faradic current in all these cases, except for the slight blurring of the angle which is found in all records of direct stimulation, however anatomically homogeneous the muscle.

The only conclusion which can be derived from these experiments is that duration of twitch, the opacity (granulation) of fibre, and the size of fibre, all may vary independently. The variations in the twitch durations of soleus and gastrocnemius from one animal to another can be quite large, and the following list (in σ) is of paired muscles from a number of animals in fair condition (neither emaciated nor fat), and the twitch duration of soleus in each pair is enclosed in brackets:—100 : (260), 40 : (120), 60 : (180), 48 : (320), 65 : (225), 45 : (200), 76 : (200), 40 : (170). These twitches were all recorded rapidly after fixation of the preparation, to obviate the lengthening of twitch duration caused by cooling. A record of a twitch from one cold soleus (approximately 23° C.) was 700 σ in duration. Soleus has been found as rapid as 100 σ in twitch duration, and so it is seen that although gastrocnemius may have a longer twitch duration than some examples of soleus, these muscles are always arranged in the same animal so that gastrocnemius is the more rapid.

The function of the granules or “liposomes” admits of some speculation. It is of especial interest to find if it has any bearing on fatigue. Gastrocnemius and soleus on one side were fatigued by a series of break shocks at 20 a second with the blood supply cut off (mammalian muscle is quite difficult to fatigue when the blood supply is good). After fatigue from indirect stimulation strong currents were applied directly to the muscle until no response occurred, and the muscle was then examined by the Sudan method, with the muscles of the other limb as control. No difference could be detected. This confirms Bell (6) who could find no evidence of disappearance or change in the liposomes in frog muscle after fatigue. The differences in the fatigue period in gastrocnemius (pale) and soleus (red) in the cat are but little marked in my experiments, as was also found by Lee, Guenther, and Meleney (soleus, extensor longus digitorum, tibialis anticus, and diaphragm) (50), in contrast to those of the rabbit, observed by Ranvier (66) and Hay (35).

In the *new-born kitten* all muscles are opaque (heavily packed with large granules) the fibres are very small in diameter, and the tension response insignificant. A motor twitch from gastrocnemius in a decerebrate 5-day old kitten was recorded by a delicate mirror myograph (of pattern described by Sherrington). A tracing is reproduced in text-fig. 4, a, where the duration is seen to be 220σ . Transverse sections of this muscle revealed (fig. 12, Plate 22) that all fibres are extremely small, varying very little from 0.0001 sq. mm., and the heavy granulation is apparent. The granulation even at this early stage is aligned with the cross striation and runs evenly the whole length of the fibre. Soleus in this animal did not give a recognisable twitch but the after-action of a tetanus (some 2 grams tension) was 320σ . The cross section appearance was exactly the same as that of gastrocnemius, except that 50 per cent. of the fibres were a little larger in area (0.00022 sq. mm.).

Meyer (see Banu (2)) showed that the development of rapidity of contraction in the cat took place after birth, and recorded the slow contractions of the new-born and 7-day kitten. Banu (2) has shown that the development of rapid contraction is paralleled by the development of shorter chronaxie. With the object of tracing the development of the "liposome" fibre-pattern, so marked in the adult gastrocnemius, older kittens were myographed. At 14 days the pattern has completely differentiated. Gastrocnemius in this animal was 340σ in twitch-duration (text-fig. 4, b) and the cross-section revealed (figs. 13 and 14, Plate 22) that a proportion of fibres had become completely clear. The largest fibres were 0.00022 sq. mm. in diameter and were clear of granules; the smallest fibres contained both clear fibres (22.3 per cent.) and dark fibres (11.0 per cent.) and averaged 0.0001 sq. mm. A great many intermediate types existed, but the large clear type provided 19.1 per cent. of the fibres, and, this being the case, it was of interest to find that the motor nerve tetanus showed an after-action in which no early drop could be found (text-fig. 4, c) until the angle, which occurred at 220σ after the end of the stimulus.

Soleus in the 14-day kitten developed a small tension, of duration 260σ (text-fig. 4, d), thus being shorter in duration than gastrocnemius. It is the only case encountered so far where soleus has shown a shorter twitch duration than gastrocnemius in the same animal. Whether this is always the case at this stage cannot be stated, as no other animals of that age were available. The after-action of a tetanus in this soleus lasted 260σ and showed no preliminary drop (text-fig. 4, e). Since the after-action of a tetanus in gastrocnemius was shorter than that of soleus, it may be that the relatively long twitch in the former muscle is an indication of some process preliminary to differentia-

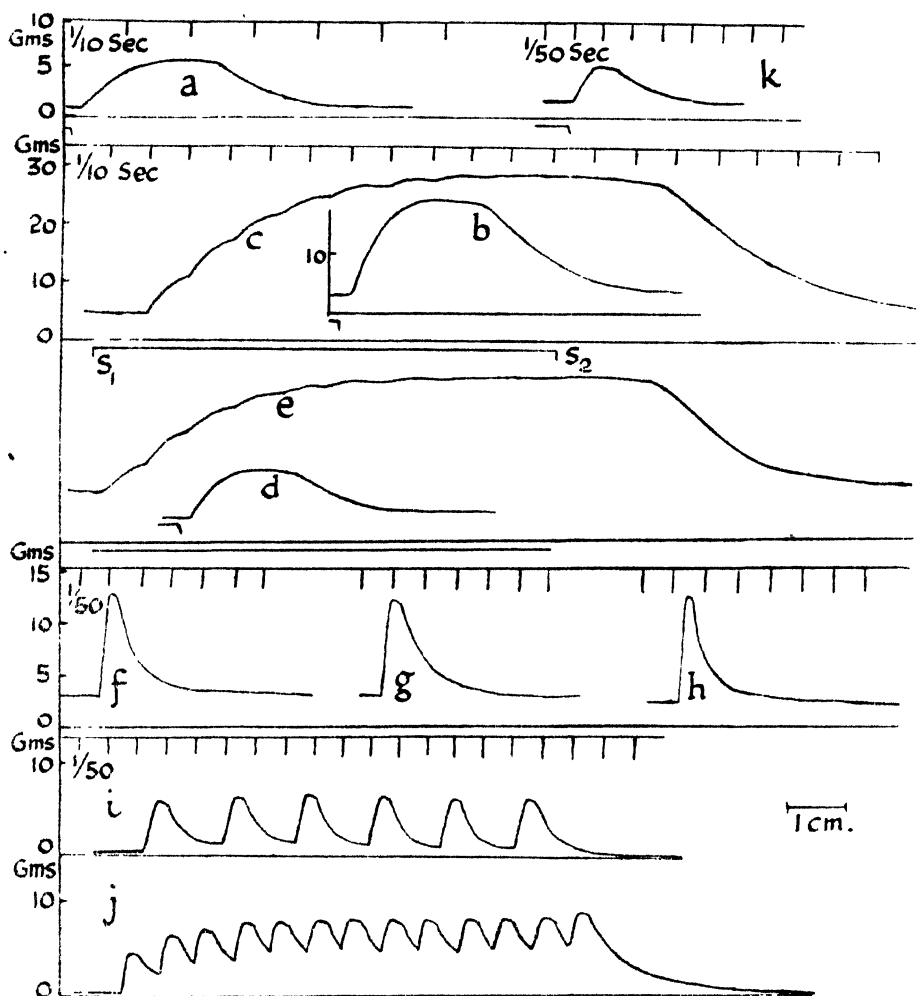


FIG. 4—(a) Motor twitch of *M. Gastrocnemius* of 5-day kitten (break shock stimulus to sciatic nerve at end of lower line). (b) Motor twitch of *M. Gastrocnemius* of 14-day kitten. (c) Motor tetanus of the same muscle, higher initial tension. Stimulus rate 8 a second (on at s_1 , off at s_2). (d) Motor twitch of *M. Soleus* of 14-day kitten. (e) Motor tetanus of this soleus at higher initial tension and same rate of break shocks as in (c). Time and tension for (b), (d) and (e) as in (c). (f) *M. Internus Rectus*. Motor twitch by break shock stimulus to n III intracranially. (g) The motor twitch of the same muscle in response to a strong break shock applied directly to the muscle by means of two pins. (h) *M. Internus Rectus*. Twitch response of great speed in response to break shock to n III intracranially. (i) *M. Superior Rectus*. A series of break shocks applied to n III intracranially at a rate of 20 a second. (j) As (i), but rate of break shocks 40 a second. (k) *M. Retractor Bulbi*, superior slip. Twitch contraction in response to a break shock applied directly.

tion of duration, a process which did not affect after-action. The histological picture of this soleus reveals (fig. 15, Plate 22) a differentiation of pale and dark fibres similar to that in gastrocnemius, but the fibres are all appreciably larger, the great majority averaging 0.0003 sq. mm. with an appreciable number of fibres of 0.00022 sq. mm., but very few indeed as small as 0.0001 sq. mm. The light fibres are practically all of the large variety and are in fewer numbers than in gastrocnemius.

From the responses from the muscles of the 14-day kitten it is apparent that light, clear fibre does not confer rapidity of duration. The relative rapidity in duration of gastrocnemius begins to make its appearance from the late third to the fifth week, and an animal between the fifth and sixth weeks gave twitch-durations of 40 σ for gastrocnemius to 170 σ for soleus. At the end of the fourth week the twitch of gastrocnemius shows an early angle (50 σ) and early and late delays in relaxation, signs evident of variation in duration. Soleus in this animal was 130 σ in duration. This was evidently during the period of change in duration in gastrocnemius. Thus in the 14-day kitten the interval between the appearance of non-granulation and of speed of contraction has been struck, and the first clearly makes its appearance before the second.

In the course of appearance of clearness it is among the fibres situated on the outside of a fasciculus that the change is first seen, and especially in those fasciculi which pass in the neighbourhood of large vessels. This is not due to any direct relationship to the interfascicular fat, or to the vessels, because in longitudinal section the whole of a fibre is pale, far away from where the vessels pass over it, and also because one very dark fibre may exist (dark throughout its length) embedded in a small collection of such pale fibres. It may mean that the regulation of the arrangement of pale fibres is caused by the vessels and that the capillary area is more dense near these vessels. The arrangement of clear fibres on the outer edge of the muscle fasciculus is prominent in the pectoral muscle of the pigeon (figs. 16 and 17, Plate 22) and is occasionally seen as a modified palisading in adult cat muscle. In all these unstained preparations the capillaries, when cut in cross-section, are quite clear. The clear fibre possesses no special arrangement of such capillaries, nor does it possess any particular transverse arrangement when seen in longitudinal plane in Bielchowsky preparations; both types of fibre appear exactly alike in vascular arrangement.

It has long been known that the external ocular muscles were composed of fibres of smaller size than those of the remainder of the body musculature. Halban (33) further noted that in a man with great muscular development the

eye muscle fibre remained unchanged, although the diaphragm and other muscle was found to have increased fibre size. From what has been described above it is seen that fibre size may vary independently of contraction-duration. Therefore it is of interest to examine the twitch of external ocular muscles for twitch duration and tetanus after-action. The motor twitch of any external ocular muscle in the cat is a feeble, rapid affair, and the sensitive myograph used for the muscles of kittens was used to record it. Owing to the recording system being on the principle of a light beam moving on a dark field, it has not yet been possible to register the action current as well. The duration is therefore measured as in the curves from kitten muscle, from the mechanical onset to the angle. A slight correction (about + 6 per cent.) is required to compare this with the twitch durations measured from the onset of the action current. The following contraction-durations are uncorrected.

The internal rectus of a cat showed a contraction-duration of 12.0σ to a break shock applied to the intracranial portion of *n. III* (also after maximal direct stimulation) (text-fig. 4, f, g) and contained no fibre of greater sectional area than 0.0015 sq. mm. and many dark fibres of 0.0004 sq. mm. (fig. 18, Plate 22). Furthermore, the twitch has relaxed to a negligible tension within 60σ of the stimulus, direct or indirect, weak or strong, and yet there are some 15 per cent. of small dark fibres of the kind called "red" or "tonic" by various authors. All the external ocular muscles were termed "red" by early workers because of their small fibre size and granulation, but the Sudan method shows the granulation to be just as in a rapid muscle elsewhere. The twitch duration of internal or superior rectus is often below 10.0σ and one internal rectus gave twitches of 8.5σ duration. A twitch of internal rectus of duration 8.8σ is shown in text-fig. 4, h (stimulation of cut *n. III* intracranially). Compared with the latent period of a cooled soleus, which can be 5.2σ from a break shock to the popliteal in the thigh, this twitch duration in internal rectus is remarkable. It is without doubt an indication that the two processes, nerve excitation and muscle excitation, need not be considered different because of difference in speed.

M. Superior Rectus shows but little difference from internal rectus, being usually a shade longer in duration (1.2σ) and possessing exactly the same fibre arrangement and proportion of dark and light fibres. A superior rectus giving to direct stimulation maximal twitches lasting 15.1σ showed little fusion at a rate of stimulus of 40 a second (text-fig. 4, j) and at half that rate there was no summation at all (i). Summation was not any easier with stimuli, direct or

indirect, which were strong enough to escape to the fifth nerve, and therefore the presence of slower elements is unlikely. Summation of the rapid twitches, when the rate is sufficiently rapid, reveals that in these muscles of very short duration the tension ratio of twitch to tetanus is as low as in red muscle.

Similarly *M. Retractor Bulbi* in the cat occurring as fine slips innervated by *n. VI*, and seen to cause by its total contraction the quick movement of retraction of the globe and outward displacement of the nictitating plate, and noted by others (41) to contract in separate slips (each corresponding to an external ocular muscle) in the movements of nystagmus, is a muscle of fibre-type similar to the other ocular muscles, except that it possesses very few dark fibres. In the animal which gave the twitch and tetanus from superior rectus described above, retractor bulbi in response to direct stimulation gave a small twitch of 30 σ duration (text-fig. 4, k). This is considerably longer than superior rectus, but that there are no slower fibres is indicated by the slight amount of fusion found in a tetanus from supramaximal stimuli at 20 a second.

M. Pectoralis of the pigeon in transverse section shows a high content of fine granular fibre arranged in bundles, each having a few scattered large pale fibres arranged in palisade formation around the periphery. Any portion of the muscle stimulated directly or indirectly by maximal break shock (decerebrate preparation) gives a rapid twitch curve of 60-75 σ duration (text-fig. 5, a [60 σ]). A transverse section of the muscle giving this twitch is shown in figs. 16 and 17 Plate 22, and a fibre count gave 94.3 per cent. of granular fine fibres of 0.0009 sq. mm. and less, and 5.7 per cent. of clear thick fibres. There were no intermediate fibres in the portion of muscle myographed, the clear large fibres varying between 0.0036 and 0.0072 sq. mm. Thus the muscle twitch curve reaches one-seventh of the twitch tension 120 σ after excitation by maximal stimulus, direct or indirect. The motor tetanus shows no evidence of slow fibre (text-fig. 5, b). If the fast twitch be the expression only of the pale fibres, the small dark fibres must be totally inexcitable not to show more effect, since they are present in such large amount. The motor tetanus shows no evidence of slow after-action. It is inconceivable that the powerful twitch and tetanus from the muscle is produced by the pale large fibres only: therefore it must be concluded that each type of fibre in this muscle has the same duration in peripheral contraction. Similarly the superficial calf muscles in the pigeon's leg show a twitch duration of 55 σ . The deep calf muscle gave a twitch also exactly 55 σ in duration, and in cross-section the fibres of the two were indistinguishable, and appeared intermediate between the large and small fibres of the pectoral muscle of the same animal. Of these two calf muscles the

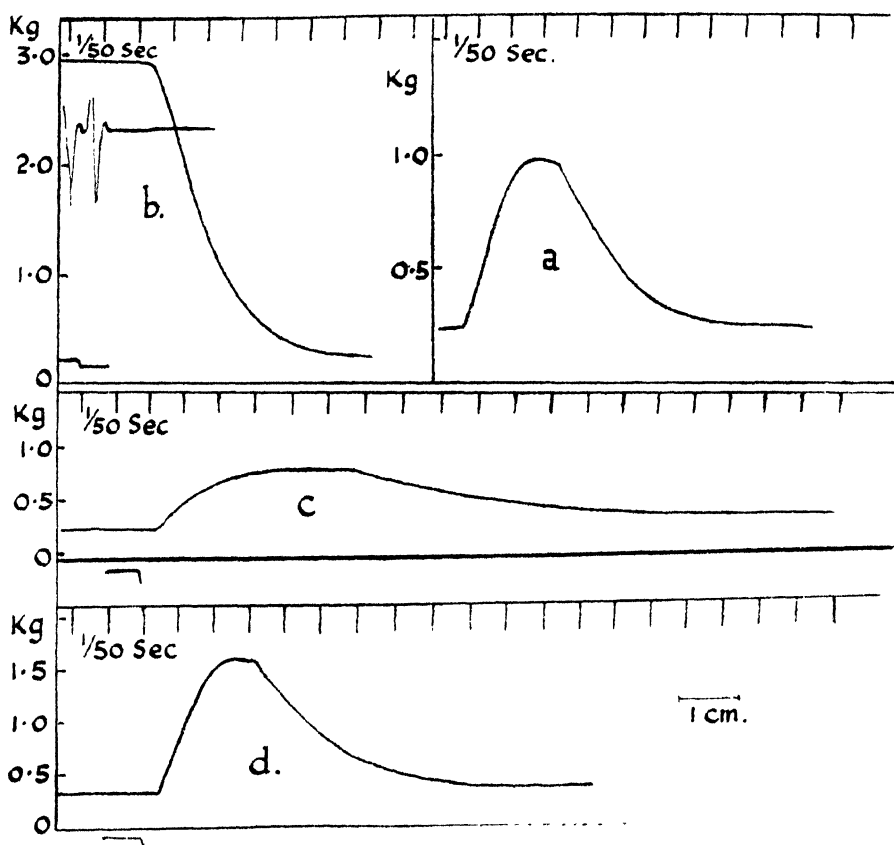


FIG. 5—(a) *M. Pectoralis* Pigeon. Twitch response of the anterior sternal portion in response to a single break shock to the motor nerve. (b) The same muscle recording in (a), but here at the termination of a motor tetanus at 50 a second. (c) *M. Soleus*.—*Macacus Rhesus*—Ether anaesthesia. Motor twitch in response to a break shock to the sciatic nerve. (d) *M. Gastrocnemius*, the same preparation from which (c) was obtained. Motor twitch in response to break shock to sciatic nerve. The action currents are simple and brief for each twitch (omitted from a, c, and d, in tracing).

superficial has a lighter colour than the deeper, and both are very much paler than the deeply pigmented pectoralis.

The complexity and variability of the substance composing the granules of opaque fibres is indicated not only by the variable effects of osmic acid, but also by differences of staining between an opaque fibre and a clear fibre, even after dehydration in alcohol and clearing in xylol or benzol. It is possible, of course, that something remains even after all the substance staining with Sudan, osmic, hæmatoxylin, etc., is removed by starvation, but if so there is

no way of appreciating it by ordinary histological means. The conclusion is inevitable that the "dark, granular" appearance of the early histologists has no significance in terms of contraction, but is in some way connected with nutrition.

In order to discover if any of these granular fibres were dependent upon some nerve stimulus for their lipoid-storing property, since they were so sharply divided from neighbouring fibres, the effect of section of various possible sources of supply was observed. Animals were studied in which ventral and dorsal roots had been sectioned for two weeks, others in which the sympathetic chain and lumbar and first sacral ganglia had been excised for a fortnight, and for much longer, and others with the dorsal root ganglia excised for the same periods. In all these the distribution and depth of staining of dark and light muscle fibres in gastrocnemius and soleus remained the same on the operated side as on the control side. So too, no obvious differences in diameter were found, except in those experiments in which the ventral root was sectioned, in which case fibre diameter lessened in proportion to the wasting. The events in disuse atrophy in the other cases were avoided by the short period after nerve section, and no change was then seen, although evident after section of the ventral root for the same period. It must be admitted that the property of lipoid storage or protein storage is a characteristic of the muscle cell, and although the remaining muscles enlarge with feeding, some, for example, the eye muscle fibres, do not enlarge. This nutritional property remains even if all the nerves be cut, or if the sympathetic be left alone intact, or if the sympathetic alone be cut. If the sympathetic have a trophic, or metabolic effect upon muscle, whereby it directs or orders the protein metabolism or fat metabolism, then such an influence is so slow in effect that 14 days of lack of stimulus have no obvious result. A normal animal, after excision of the lumbar sympathetic on one side, became quite thin within 10 days: but when it was then killed, the muscles on both sides were equally clear, so that mobilisation of the fat does not require a stimulus from sympathetic efferents.

The duration of the motor twitch of both slow and rapid muscles occasionally alters to become as much as 10 per cent. longer than that of the control side after excision of the sympathetic ganglionic supply to the limb; but this effect is inconstant, often reversed within a few minutes, and is unaffected by subsequent stimulation of the preganglionic fibres, when only these fibres have been interrupted. The change occurs within a few minutes and is not progressive, either no change or but a very slight one remaining after two weeks have elapsed after excision of the ganglia.

Two fibres lying together in a particular muscle "head" may therefore be totally different in their properties for accumulating lipid, or other substances, but may possess approximately the same speed in contraction. Lipoid storage and protein accumulation are factors which, though not concerned with the speed of contraction, appear to be most specialised in fibres which are most specialised for contraction speed. The pale fibres in gastrocnemius in a fat cat do not appear able to accumulate fat, however much is offered them by the blood stream, for they appear to have lost the capacity for storing lipoidal granules and are concerned in accumulation of other substances tending to make the fibre more bulky, while in soleus the fibres may become opaque, or bulky, or both. It is a noticeable feature of the intrafusal fibres of the muscle spindle that they are always clear and thin. Even in the 5-day kitten the intrafusal fibre has become clear, and so it thereafter remains, free from the encumbrance of fat accumulation, and not liable to increase or decrease in size. The eye muscles similarly appear free from large changes in size, but other factors not affecting size, but concerned in the accumulation of liposomes, are clearly present, and are made evident by the small dark and the slightly light fibre.

It must be concluded, therefore, that there exists in *M. Soleus* in the cat only one class of fibre, as far as duration of the peripheral contraction process is concerned. That class may vary within itself, but the smooth tension curve leaves a strong impression that the maximum and minimum of the variations from the group-response are very small indeed, and variation in duration of response, when it occurs, must involve all fibres. The duration of the process is not in any way dependent upon the diameter of the fibre or upon the opacity of the fibre, while the length of all the fibres is approximately equal. In the same way, the internal head of gastrocnemius is of simple contraction-duration, but much more rapid than soleus. In quadriceps, the red crureus corresponds in every respect to soleus, except that it is shorter in twitch duration in the same animal. The pale, rapid heads of quadriceps and other muscles present the histological appearance of gastrocnemius in the same animal.

The flexor muscles are all similar to the pale extensors in duration, often a little more rapid, sometimes a little more slow, and the appearance of their fibres in cross-section is the same. They are, in the cat, all pale, and rapid in contraction, and also in the rabbit; for the red, slow, *M. "Semitendinosus"* (Krause (47)) in the latter animal behaves as an extensor, contracting reciprocally with the pale "*semimembranosus*" in the hopping movement in the decerebrate animal. The latter muscle, inserted into the crest of the tibia,

is the same as the semitendinosus in the cat (Mivart (58)), an undoubted flexor, and it seems that here Krause was in error in calling it "semitendinosus," for the red muscle in the thigh of the rabbit is identical in origin and insertion with a portion of semimembranosus in all animals, and indeed Lesbre (51) suggested that it be called semimembranosus. The red muscle in the thigh of the guinea-pig appears to be a portion of gracilis.

(c) Red Pigmentation in Relation to the Contraction Process.

Soleus, crureus, and the inner short head of triceps brachii, in the cat are always of a deeper red than their more rapid associated muscles, and this is so also in the rabbit. This colour difference is even more marked after washing out the vessels, when very little colour is left in any of the rapid muscles (even when the latter have a high content of dark fibre). Bichat (8) pointed out that in an animal killed by asphyxia, the muscle pigment would still be light red, while the blood in the vessels was dark. Investigations have tended to show the presence of two pigments in muscle (MacMunn (56), see also (39)) the one a hæmoglobin and the other a "myohæmatin" or "cytochrome." Pectoralis in the pigeon contains both pigments, yet the very dark red pectoral of the pigeon is as rapid in contraction as the pale unpigmented ankle extensor of the same bird ; so that in this animal the red muscle pigment is definitely not associated with great slowness of contraction.

Further, in the 14-day kitten, as described above, both gastrocnemius and soleus are slowly contracting, yet both muscles lack red pigment (though soleus is a little darker than the other), while the intrinsic muscles of the foot are then deep red. Redness of muscle in the adult cat is a fairly reliable guide to slowness of contraction, but a crureus is often as red as the soleus, and yet shows only a quarter of the twitch duration of that soleus.

The pigment does not stain with eosin or iron hæmatoxylin, although these stains differentiate between the clear and dark fibres in a pale muscle. The suggestion of Whipple and Robschey-Robbins (78), that muscle (as well as the liver, kidney, and spleen) can put aside a hæmoglobin precursor, seems to lie nearer the solution of the problem.

Although red pigmentation is so closely allied with slowness of contraction, there is reason to believe that this is a chance association and not a necessary interrelation. The necessity for the red pigment in red muscles, owing to their apparent association with maintained contraction, has been advanced as a reason for its presence as a catalyst (Needham (60)) and an oxygen carrier. As will be seen later, there is no reason to suppose that red muscle ever con-

tracts without blood flow any more than does pale muscle, and therefore should need no more assistance in oxidation.

(d) The Amount of Sarcoplasm.

Paukul (61) showed that the areas of Cohnheim are more evenly distributed in red than in the pale muscle, and Rollett (71) showed that the dark striped muscle fibre of the bat contained large areas between and around the fibrils and were slow in contraction. In the material examined in the present investigation any matter staining deeply with iron hæmatoxylin or hæmatoxylin and eosin, and lying between the fibril bundles, seemed to be more in the nature of envelopes or débris of the granules after dehydration in the higher alcohols. The coarse areas in pale muscle are the more evident the less carefully fixed and dehydrated. Pale muscle is more difficult to cut in frozen section, more fragile than red, partly on account of a smaller content of connective tissue, as noticed by Arloing and Lavocat (1), and partly from the nature of the fibre, the areas of Cohnheim being more marked in the large pale fibres. Either of these areas are either small artefacts, or fibre-splitting begins at these boundaries. The myofibrils are tightly packed when the large liposomes are present.

The wing muscle of certain insects possesses an amount of sarcoplasm which is great enough to obscure the fibrils themselves; yet the wing contraction in these insects is very rapid. The sluggish larvæ of the same insects possess muscles containing very little sarcoplasm (76). Bottazzi (9, 10) argues that the long response of red muscle is due to its great content in sarcoplasm. It is evident that this cannot be true for the slow muscle fibre of soleus in the cat, in which the "sarcoplasm" is scanty, unless we assume that the myofibrils, as numerous here as in a rapid fibre, are in this case entirely inactive.

(e) The Vascularity of Red Muscle.

It was shown long ago by Ranvier (65) that the red muscle of the rabbit possessed dilatations on the capillaries. In the large number of Bielchowsky preparations made for the study of the nerve endings in soleus, gastrocnemius, and other muscles, the capillaries are generally most clearly seen. A similar dilatation, capillary or venous, was never once seen in soleus of the cat, and no difference was detected between the capillaries of red and of pale muscle in their disposition, wall, or termination. Ranvier considered that these capillary dilatations were adapted to the slow contraction to enable the blood to pool in the muscle during contraction, but this, when the velocity of blood flow in the capillary and the size of the dilatations (0.017 to 0.025 mm. diameter),

are considered, could account for very little blood. As a reservoir they could contain approximately the number of corpuscles in one length of capillary.

As shown by Krogh (46) the capillaries of muscle all dilate with activity of the muscle, and soleus in the cat was watched under the microscope (15) (oblique illumination by a 100-c.p. lamp) during the active tension of a stretch reflex and during rest and no interruption of the blood flow was ever seen. On the contrary in reflex tension of over a kilogram (probably more than necessary to support maintained extension of the ankle against the weight of one hind-quarter of the animal) the blood flow near the surface of the muscle and on the surface was seen to be quite extraordinary. This was seen in quite deep vessels, and there is no doubt that active increase in the blood flow occurs and is sustained. In a maximal isometric motor tetanus some hindrance of the blood flow does occur according to Claude Bernard (7), Burton-Opitz (13), and others, and a resulting increased flow with relaxation, but it is practically certain that a maximal tetanus of rapid rate of stimulation never occurs in soleus under natural conditions.

This agrees with the finding of Dusser de Barenne and Burger (17), that the respiratory exchange is increased in static effort, and therefore the blood flow to muscles is then not impaired.

(f) The Influence of Length of Fibre, and of Shortening, upon Contraction.

It is of interest to compare the length of fibre in slow and in rapid muscle. The maximal twitch of a soleus of an average fasciculus-length (= fibre-length) of 1.45 cm. was 280σ ; the companion gastrocnemius was 1.05 cm. in average fasciculus-length and 65σ in twitch duration. At the rate of wave propagation assumed for mammalian muscle, the difference in average natural fasciculus lengths does not seem adequate to account for the difference in twitch durations.

In any particular muscle the influence of passive fibre-length (initial tension), as Fulton (20, 25) demonstrated, is a marked one. Increase of initial tension within physiological limits in frog and cat muscle increases the duration of the isometric response and the tension of the plateau. For example, in one animal, gastrocnemius (both heads) at a length (total) of 9.8 cm. exerted an initial resting tension of 50 grams, and a twitch developed 950 grams and was 60σ in duration. Increasing the length to 10.55 cm. raised the resting tension to 180 grams and the twitch then developed 2400 grams and lasted 70σ . In the same animal, soleus at 9.3 cm. length (total) exerted 40 grams of initial tension, and a twitch developed 160 grams and lasted for 220σ . At 10.50 cm. length the initial tension was 80 grams and the twitch tension was 270 grams,

while the duration was now 280 σ . The duration in both types therefore varies with length, but, whatever the initial length, soleus never has a duration approaching that of gastrocnemius.

As was worked out by Fulton (24) for frog muscle, shortening, allowed during the motor twitch, causes a decrease in the duration. By allowing varying degrees of shortening to soleus it was found that alteration of the shortening from 0.04 mm. through intermediate values to 0.7 mm. caused a variation of from 280 σ to 265 σ in one muscle.

There is a minimum duration for soleus, and that minimum is reached with the allowance of comparatively little shortening and slight initial tension. The muscle cited above varied from 200 to 280 σ in duration, and would not relax under 200 σ whatever the shortening allowed, and would not be longer than 300 σ in duration even with the maximum initial tension and the smallest amount of shortening available. Thus soleus in the adult cat has a *range* in duration which is always remote from the range of durations in gastrocnemius. The gastrocnemius of the animal, from which this example of soleus was taken, showed a similar range from 79 σ minimum shortening and maximum initial tension, to 65 σ with maximum shortening and minimum initial tension. A soleus of range 200-280 σ is thus associated with a gastrocnemius of range 65-79 σ . It has never been found that the *ranges* of these two associated muscles in an adult cat have overlapped. The figures given earlier (p. 390) for soleus and gastrocnemius and soleus are all for maximum duration, as also are all others mentioned before this. Both length of fibre and ability to shorten may therefore be excluded as reasons for the relatively long twitch duration in soleus.

(g) *The Distribution of Slow Muscle.*

It is now possible to say that *there is no histological criterion of the speed of contraction of a muscle fibre*. It has been shown that soleus in the cat is for practical purposes all composed of slowly contracting fibre and gastrocnemius of rapidly contracting fibre. Plantaris was examined in some preparations and found to be but little longer in twitch duration than gastrocnemius. In one case gastrocnemius averaged 50 σ in maximum duration, plantaris 55 σ and soleus 240 σ . The duration ranges of plantaris and gastrocnemius overlap, but neither approaches the range of soleus. The external head of gastrocnemius is often much shorter in duration than the internal head (*e.g.*, 60 : 85 σ). Similarly the short medial head of triceps brachii is composed of slow fibre (though always more rapid than soleus in the same animal) and the short

lateral head of more rapid fibre. In the quadriceps of the cat, crureus is always slower than vastus externus, although like the medial head of triceps it is more rapid than soleus in the same animal. The relative speeds of contraction are not always the same, but the medial, short head of triceps, the crureus, and the soleus are always slower than their associates. In the rabbit also this is the case, and it is interesting, in view of Grützner's theory of muscle-mixture in higher mammalia, to examine the muscles of the monkey. In one *Macacus Rhesus* myographed, the soleus twitch was 125σ in duration (text-fig. 5, c), the gastrocnemius twitch 60σ in duration (text-fig. 5, d), and each showed the typical, simple curve found in the cat. Similarly the medial, short head of triceps in another *M. Rhesus* was 80σ in twitch duration and the lateral short head was 55σ . So, too, in man the same muscles probably preserve a relative difference in contraction-duration, for thus the muscles of higher mammals are arranged as are those of the lower, and in all these complex muscle groups the deeper layer, of shorter tendon length, is always slower in contraction.

Is slow muscle always a separate "head" of a larger muscle, as is soleus for the ankle extensors? In work in collaboration with Dr. Liddell (16), on *M. Supraspinatus*, an extensor of the shoulder, two elements of different duration were encountered. These could be separated by minimal make and break shocks. A determination showed the chronaxia of the nerve for fibres of rapid duration and for fibres of slow duration to vary in relation one to the other. These chronaxia were in some few cases approximately those for gastrocnemius and soleus, but, as mentioned earlier, the slow muscle is occasionally the more excitable. In examination of a cross-section by the naked eye, supraspinatus is seen to be made up of a superficial pale layer, separated from a deep red layer by a layer of fascia, which sometimes is itself partly divided into two layers of fascia separated by a serous space. It was possible, when the maximal twitch of supraspinatus had a duration of 60σ with a delayed relaxation, to dissect the superficial layer rapidly away, and it was then found that the deep layer gave a pure response 120σ in duration.

The muscle fibres are therefore grouped according to their duration of contraction, even in *M. Supraspinatus*. Similarly "semitendinosus" (red) in the rabbit almost invariably gives a double response, even if the surrounding muscle be dissected quite away from it. Here the muscle has a small, rapid-fibre content. In the dog, pig, many hyænidæ, the kangaroo, and the otter, soleus is not present, and the function of soleus must therefore be capable of fulfilment by gastrocnemius. In the pig Meckel states that soleus is absent,

though Lesbire (51) considers that it has merely shifted its attachment to the femur. Fusion with gastrocnemius may conceivably have occurred in the dog and the others.

From the isotonic records of Bottazzi (10), and of Briscoe (11), and the detailed analysis of excitability of the "hump" by Bottazzi, it is evident that the crura of the diaphragm in the cat possessed an undoubted slow element; but from the facts already given for soleus in the cat, it is certain that the slow element in the diaphragm is not necessarily the dark, granular fibres described by Klein (40). The "nase" of Funke (26) seen in gastrocnemius of the frog, and obtained especially from fatigued muscle, is probably the result of some slower element in this muscle, and would there represent a fused soleus. The "nase" of mammalian supraspinatus (16) is certainly due to slow red muscle.

Since all the muscle of kittens at 5 to 14 days is of long contraction-duration, it is evident that in the adult cat some change in speed of contraction has occurred in muscles like gastrocnemius. From all the foregoing it would appear that the development of rapidity in contraction is a factor which, for some reason, affects the more superficial extensor muscle groups, and all the flexor groups.

The slow contraction of the retractor bulbi compared with the associated recti, and similar small variations in contraction-duration between one flexor and another, are in all probability the expression of a similar arrangement of less degree.

6. *The Influence of the Ventral Root Innervation.*

On the other hand, all muscular function is dependent upon the existence of the motor root fibre. Section of the ventral roots and prevention of regeneration ends in the ultimate disappearance of muscle as a contractile structure. The early onset of this type of degeneration is evident within a surprisingly short time. For instance, in a gastrocnemius the duration of the twitch was 90 σ (the 1st, 2nd, 3rd and 4th lumbar rami were previously sectioned), while 2 minutes after section of the sciatic it changed to 100 σ . The contraction-duration of soleus was 340 σ , and changed in that time to 350 σ . This effect is also seen well in the 14-day kitten, where the twitch from the intact sciatic is 325 σ in duration (gastrocnemius undifferentiated); about 2 minutes after section of the sciatic it was 340 σ , and approximately 1 minute later it was 360 σ . Hand in hand with this rapid increase in duration is a fall in tension, especially marked at the ascent plateau angle.

This early difference in the maximal response is seen also in the motor tetanus, and it is probably the explanation of the differences in maximal reflex

tetanus and maximal cut-motor-nerve tetanus, described by Liddell and Sherrington (53). After the first rapid fall (within 2-10 minutes), the response deteriorates relatively slowly, and under conditions of appropriate warmth and good blood supply the twitch and tetanus then reach a fairly constant value, which may remain steady for many hours. It is upon such responses that the durations in the first portion of this paper are based. The fall in duration and tension after section of the whole motor nerve is, therefore, in greater part due to section of the ventral root fibre. Deafferenting the muscle seems to have no effect in this connection.

Twenty-four hours after section of the ventral roots intradurally gastrocnemius was 55 σ in duration (nerve stimulation) compared with 50 σ for the normal side (intact nerve), while soleus was 190 σ on the sectioned side and 180 σ on the normal side. Forty-nine hours after intradural section of the ventral roots the degenerating gastrocnemius was 88 σ in twitch duration (compared with 80 σ for the normal side) and the degenerating soleus 189 σ compared with the normal side duration of 175 σ . Parallel with the changes in contraction-duration the tension developed by maximal motor tetanus is falling. After 24 hours from section of the ventral root the maximal tetanus tension of the internal head of gastrocnemius has already fallen to 83 per cent. of that of the normal side.

For some considerable time, ligature of the femoral artery is found to affect this cut-motor-nerve duration not at all, while the tension-height soon rapidly declines. Even after death, when the tension development rapidly declines, the duration of gastrocnemius is but slightly prolonged, and again in deep fatigue the twitch becomes but little longer in duration (sometimes very much shorter, for example, 100 σ to 60 σ), but the relaxation then becomes more linear instead of concave. This linear relaxation is like that of a curve of muscle of mixed speeds, and may here indicate the temporal dispersion of the angle in different fibres, as indicated by Fulton (25) for fatigue in frog muscle.

7. Discussion.

Mammalian striped muscle has been shown to be concerned in at least two functions which are not interdependent. In its primitive form each muscle fibril is packed with granules, and the process of contraction is extremely sluggish and delayed, but early in the extra-uterine life of the kitten fibre-differences begin to occur. The first of these differences observable is the loss of granular opacity in some fibres. This process rapidly progresses, and muscle becomes a mixture of clear and dark fibre. In the adult practically all

dark granular fibres can be made entirely clear by reduction of the animal to an emaciated condition. On the other hand, examination of fat animals reveals that, while the clear fibres of soleus and other "red" muscles can become granular and opaque, there is a type of clear fibre, in pale rapid muscles, which does not become granular. The process of accumulation of granules appears to be a sign of the storage of some complex lipid material.

The even distribution of clear fibres in the gastrocnemius of the 14-day kitten means that, already, this type of clear fibre has a raised threshold for granule-accumulation, and is probably the beginning of a change in these fibres to a permanently non-granular state. Similarly the remaining fibres in pale muscle can become darker, more tightly packed with granules than the muscle-fibres of red muscle, by fattening, but seem to be, most of them, relatively free from the large variations in fibre size which are apparent in the first, granule-free set, and in the fibres of red muscles. This process of granulation would appear to be of some nutritional importance, and, in the pale muscles, the grading of its function is sharply differentiated from the function of fibre enlargement. The pale muscles are, in fact, more highly differentiated. This differentiation is found in extreme degree in the intrafusal fibres of the muscle spindle, which appear to have lost both functions.

The muscle fibre of fishes (28) can accumulate or lose large amounts of protein without embarrassment of function, as far as swimming is concerned, and the variations in size of clear fibres in mammalian muscles may possibly be likewise due in great part to protein changes. On the other hand, the small tension developed by the external ocular muscles suggests that thinness of fibre is partly correlated with small tension-development in contraction, and there is conceivably such a factor in all fibre enlargement (greater number of myofibrils); but, until the same muscle can be accurately myographed, and examined histologically, before and after a period of "training," there appears to be no definite answer to this question.

It is clear that the granular storage process can have no relation to the type of process of contraction, for the duration of the contraction process is independent of the state of granulation of the muscle fibre. The muscle fibres, however, which reveal nutritional differentiation in greatest degree, also reveal the greatest development of shortening of duration from the embryonic state. They are the most highly differentiated muscle in regard to contraction-duration. Mammalian muscle, therefore, has two factors, contraction-duration and "nutritional storage," which can be independent specialisations of the one muscle fibre. Lipoid accumulation and fibre diameter (? protein

accumulation plus myofibril increase) appear to be complementary, in the sense that full specialisation in the first involves at least partial loss of the second, and full specialisation in the second involves complete loss of the first.

The more highly differentiated muscles appear not to partake in that process which requires the red muscle pigment, myohæmatin, hæmoglobin or cytochrome, and this pigment, in turn, does not appear necessary for slowness in contraction. There is no evidence to show that the redness has any relation to fatigue ; and, indeed, the static contractions of red muscle would not appear to require any especial respiratory mechanism in the muscle fibre, because of the abundant vascular flow which is then induced. It is therefore desirable to class the pigmentation of muscle as a separate factor, at present not known to be linked with any process taking place in that complex tissue.

The greater the contraction-duration the more economical the contraction becomes (34), and, therefore, it is apparent that the slow muscles (which I have shown in another place to be the low-threshold motor units for the postural reflexes (15)) remaining undifferentiated as a muscle " head " in the extensor group at each joint, are ideal effectors for postural reflexes. The possession of speed, however uneconomical the result, is a necessity for animals such as the cat, and the appearance of the highly differentiated extensors, especially the double-joint heads, fulfils that requirement. The differentiation of short contraction-duration involves the change of the process of mechanical summation, so that, for two summed excitations, the tension is summed more in the more rapid muscle, and the duration is summed more in the slower muscle. No doubt this reflects some feature of the contraction mechanism in muscle, and it is hoped that, with the improvement of stimulating electrodes and apparatus for measuring short intervals of stimulus, this relationship will be worked out fully.

The differentiation of rapidity carries with it differentiation of the nutritional processes, but the means of differentiation remains obscure.

8. Summary of Conclusions.

1. In the mammalian striped muscle groups there occur muscle fibres which differ in the speed of the contraction process. The arrangement is such that fibres of similar speed of contraction form a group which is sharply delimited from other such groups and in most situations forms a muscle " head " (such as the internal short head of triceps, or vastus externus). In the more complex muscles the deeper heads are those composed of more slowly contracting fibres.

2. In any such groups of muscle fibres histological differences between the

constituent fibres are evident, and occur mainly as differences in content of granules and in fibre diameter. Since the structure of muscle is such that each fibre is able to contribute an effect to the total isometric tension, the occurrence of these histological differences, between fibres of similar contraction process, reveals that these differences have no direct relationship with the speed and nature of the contraction process in the fibre.

3. The granulation of the striped muscle fibre varies with the nutrition of the animal and appears to be a form of storage of complex substances which can be stained by an alkaline solution of Sudan III.

4. The fibre groups which retain the primitive slowness of contraction general in the new-born animal retain for each fibre a capacity both for a certain degree of granulation and for a certain degree of enlargement in diameter. Those groups which, in the course of growth of the animal, develop a more rapid contraction process, also exhibit a distinction between the processes of granulation and the process of enlargement in diameter; so that in those groups in the adult animal the thick clear fibre and the thinner, granular, opaque fibre are sharply contrasted, the contraction-duration of the two types being the same.

5. The hypothesis of Grützner and others that the muscles of higher mammals, including man, are composed of an intimate mixture of fibres of rapid and slow contraction types, being based on their content of clear and opaque fibre, cannot be sustained. These forms, including probably man, possess the same arrangement of fibres in large groups or "heads" of uniform speed of contraction as do the lower animals, although the differences between one group and another are, at any rate in the monkey, less marked.

6. The red pigmentation of the slow, less differentiated, muscle fibre also does not appear to be essential to the slow type of contraction process, and is probably the outward sign of some function not closely related to contraction.

7. The differentiation of rapid muscles from the slower more primitive muscles affects all the events of the contraction process, so that a twitch in rapid muscle shows the more rapid occurrence of events, in the same sequence as in a twitch in slow muscle. The rapidity also affects the process of summation, so that, while in slow muscle an early second response tends to be augmented mainly in the duration of the contractile process, in rapid muscle such a response tends to be augmented mainly in tension development.

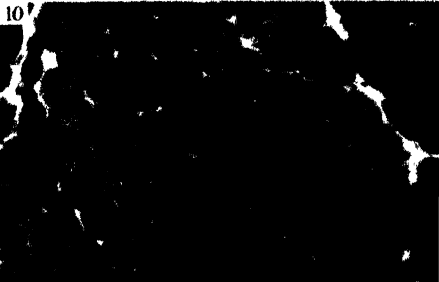
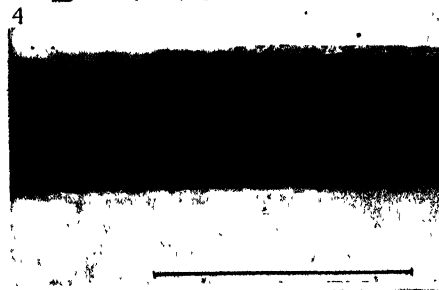
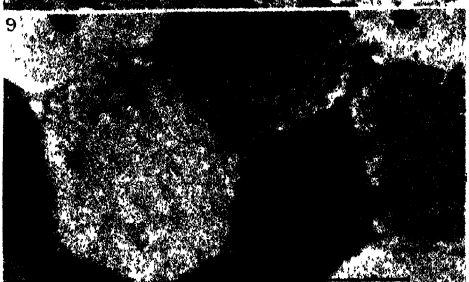
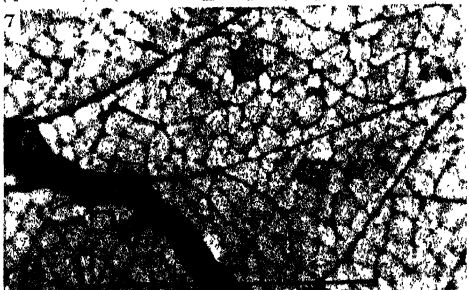
I wish to express my gratitude to the Medical Research Council for a personal grant, to the Christopher Welch Trustees for defraying the considerable cost of

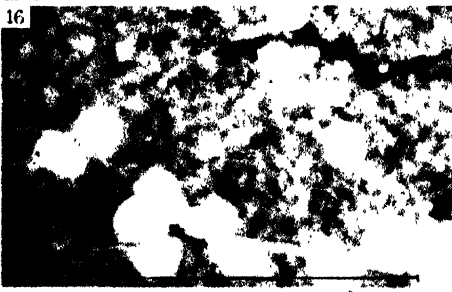
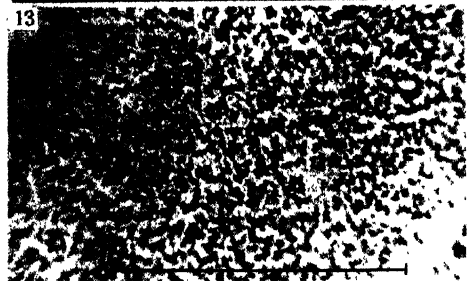
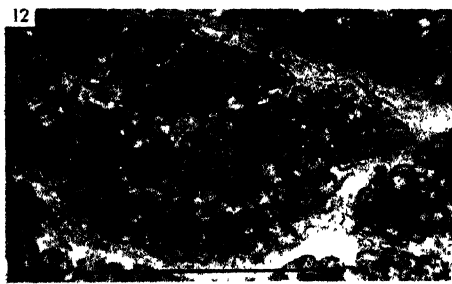
photographic material used in these researches, and to Dr. Carleton for kindly placing the facilities of his histological laboratory at my disposal.

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DESCRIPTION OF PLATES.

These figures are untouched microphotographs of sections of muscle treated by alkaline Sudan III as used by Ewald (18), in frozen sections 35 μ in thickness. The magnification is shown by means of a scale drawn on each.

PLATE 21.

- FIG. 1.—M. Soleus, Cat 1. (See table in text.) Scale length is 0.5 mm.
FIG. 2.—M. Gastrocnemius, Cat 1. Scale length 0.5 mm.
FIG. 3.—M. Soleus. Longitudinal section to show rows of granules in the dark fibre, and striation. Scale length 0.1 mm.
FIG. 4.—M. Gastrocnemius. Longitudinal section to show rows of granules in the dark fibre, and striation. Scale length 0.1 mm.
FIG. 5.—A dorsal muscle of the grass snake, to show small dark granular fibres. Scale length 0.1 mm. Nerve bundle just above centre.
FIG. 6.—M. Gastrocnemius, Cat 5 (see table in text). Scale length 0.1 mm.
FIG. 7.—M. Soleus, Cat 5 (see table in text). Scale length 0.5 mm.
FIG. 8.—M. Gastrocnemius, Cat 4 (see table in text). Scale length 0.5 mm.
FIG. 9.—As in fig. 8, higher magnification (1/6 inch). Scale length 0.1 mm.
FIG. 10.—M. Soleus, Cat 4 (see table in text). Scale length 0.5 mm.

PLATE 22.

- FIG. 11.—As in fig. 10, higher magnification (1/6 inch). Scale 0.1 mm. Showing the only thin fibre found. The clearness of this fibre controls the strain.
FIG. 12.—M. Gastrocnemius, 5-day kitten (1/6 inch) (twitch curve in text-fig. 4, a). Scale length 0.1 mm.
FIG. 13.—M. Gastrocnemius, 14-day kitten (see text-fig. 4, b and c). Scale length 0.5 mm.
FIG. 14.—As fig. 13, higher magnification (1/6 inch). Scale length 0.1 mm.
FIG. 15.—M. Soleus, 14-day kitten (see curves in text-fig. 4, d and e). Scale length 0.1 mm.
FIG. 16.—M. Pectoralis, pigeon (see curve in text-fig. 5, a and b). The outlines of the small fibres are better seen in the next figure. The clear large fibres line the contiguous edges of fasciculi of small fibres. Scale length 0.5 mm.
FIG. 17.—As fig. 16, higher magnification. The edges of two large clear fibres are seen in the lower corners. Scale length 0.1 mm.
FIG. 18.—M. Internal Rectus (see curves in text-fig. 4, f and g). Scale length 0.1 mm.
FIG. 19.—M. Soleus, Cat 6 (see table in text). Low magnification (1½ inches). Scale length 1.0 mm.
FIG. 20.—M. Soleus, Cat 2 (see table in text) (1/6 inch). Scale length 0.1 mm.

The Combination of Proteins, Amino-Acids, &c., with Acids and Alkalis. Part II.—Titration Curves of Amino-Acids in Presence of Formaldehyde.

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(Communicated by Sir F. Hopkins, F.R.S.—Received November 12, 1928.)

(From the Biochemical School, and the Nutritional Laboratory, Cambridge.)

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GROUND'S FOR AN INVESTIGATION.

1. A detailed examination of the acid-base equilibrium and the titration curves of amino-acids in aqueous solution has already been made (1), but data have hitherto been lacking for these solutions in the presence of formaldehyde, *i.e.*, relating to the methylene derivatives of the amino-acids.

2. The addition of formaldehyde to amino-acids and the resulting increase in their acidity towards phenol phthalein (or thymol phthalein) constitutes the basis of the well-known Sørensen ("formol") method (2) for their estimation by titration with soda; accordingly some knowledge of the acid-base constants and the titration curves of the systems in question is necessary before one can formulate the conditions (*viz.*, the concentrations of formaldehyde and amino-acids, and the initial and final p_H readings, etc.) under which this estimation may be carried out with maximum accuracy.

3. The acquisition of these data will permit one to explain the rationale, from the point of view of the Theory of Titration, of the Sørensen method, which had sometimes in the past received erroneous and conflicting explanations. The method was predicted by the present writer (3) to depend on the formation of a methylene derivative (or, more correctly, an equilibrium mixture of methylene derivative plus amino-acid) having an apparent p_k constant (behaviour with NaOH towards indicators) three units smaller than that of the corresponding amino-acid alone—a view which was subsequently, in a preliminary* paper (4), briefly reported to have been found accurate.

* The detailed presentation of these and the other results recorded in the preliminary paper have been delayed owing to 3½ years' absence from academic work.

4. Acid-base titration measurements in media other than water are of importance from the standpoint of the theory of titrations, and few have hitherto been made. Some unsuspected analogies appeared in the course of this work, and direct experimental evidence has been obtained in support of the "zwitterion" theory of amino-acid structure.

NOMENCLATURE.

In order the more readily to present a simplified but only approximate statement of the fundamental relations observed in the experimental work, and since the values for the activity coefficients of the system are mostly unknown, ionic concentrations will be used throughout instead of ionic activities; thus:

- (1) p_H refers to "hydrogen ion concentration" as approximately determined from indicator virage, the more theoretically accurate conception of p_{aH} , being for the present purpose neglected; and
- (2) α denotes degree of ionisation on the lines of the classical theory, in place of γ for the activity coefficient.
- (3) p_{k_a} and p_{k_b} are employed for the older and more customary "apparent acidic" and "apparent basic" dissociation constants of the amino-acid defining the effect of adding NaOH and HCl respectively as per equation (1) below, and in place of the newer *zwitterion* constants of Bjerrum (5) p_{K_A} and p_{K_B} which on the other hand define the effect of adding HCl and NaOH respectively as per equation (2).



- (4) *Determination of p_{k_a} and p_{k_b} values from titration curves.**—From the p_H found to result after a measured quantity of standard NaOH or HCl has been added to the weak acid or base respectively, these constants can be calculated by the well-known (slightly modified) Henderson-Hasselbalch equations,

$$p_H = p_{k_a} + \log \frac{\alpha}{(1 - \alpha)},$$

and

$$p_H = p_{K_w} - p_{k_b} + \log \frac{(1 - \alpha)}{\alpha},$$

* Constants are throughout expressed in the more convenient exponential form.

where α = fraction of one equivalent of NaOH or HCl (corrected) added. But the titration curve (combination curve) of a weak acid is seen from these formulæ to be identical with the back-titration curve (replacement curve) of a weak base, and *vice versa*, whenever $p_{k_a} = p_{K_w} - p_{k_b}$ (opposite signs being assigned to the NaOH or HCl added). Therefore, in accordance with recent practice, a value p_k , fixing the magnitude of the dissociation constant, may be substituted for either p_{k_a} or $p_{K_w} - p_{k_b}$, for a weak acid or base respectively, without actually defining it as one or the other.

*Titration Dissociation Constants for Di- (or Poly-) Acids, Bases, Ampholytes.**

$p_{k_{a_1}}, p_{k_{b_1}}, p_{k_{a_2}},$ etc.). The variety of dissociation constant denoted by these symbols, while approaching very closely in actual numerical value to that given by the classical definition of the dissociation constant (which refers not to the separate acidic or basic groups but to changes in the net number of ionic charges†) is, more strictly, the "titration dissociation constant," which corresponds rather with the conception of a "specific dissociation constant" for each successive group. Such constants are determined from the titration curve by regarding any substance having more than one titratable group as though it were a mixture of two or more single substances, the constant for each of which is calculated separately and independently.‡ These constants I have already used in the previous papers

* The new nomenclature of the Union of Pure and Applied Chemistry ('Bul. Soc. Chim.,' vol. 43, p. 289 (1928)) is employed in the following pages, the terms "di-acid," "mono-acid," "poly-base," . . . being used in place of "dibasic acid," . . ., etc.

† That is to say, the classical dissociation constant for the 3rd (*e.g.*) step of dissociation of a tribasic acid AH (whose three constants may be of similar magnitude) does *not*, as



it is sometimes supposed to, define the equilibrium between the ion A^- and the ion A^- ;



but it defines the equilibrium between all ions having two net charges and all ions having three net charges ; *i.e.*, between A^- , A^- , and AH on the one hand, and A^- on the other



hand.

‡ Without in any way entering into their theoretical basis, I employed these constants for calculating the titration curves of amino-acids, polypeptides, poly-acids and bases (1923, *loc. cit.* (1), pp. 441, 473, 481), and conversely I compared the values of these constants (derived from titration data) with the formerly known constants (from conductivity, etc.) (1923, *loc. cit.* (1), pp. 464, 470). Later I made use of the conception again in relation

(there called “dissociation constants from titration data”), but their use appears to me to have several important practical and theoretical advantages which will be discussed elsewhere.

p_k , is also used to denote the apparent dissociation constants (*i.e.*, calculated from indicator virages by the Henderson-Hasselbalch equation) for titrations of amino-acids in presence of formaldehyde.

I.—TITRATION CURVES AT CONSTANT FORMALDEHYDE CONCENTRATION.

SØRENSEN (2), in his volumetric method for estimating amino-acids in presence of formaldehyde, brought them with soda to a constant p_H end-point of 9 or 9.7. He found it necessary to have the formaldehyde concentration approaching 12 per cent. in order that the full titre of soda (one unit equivalent) might be attained.

J. H. BROWN appears to be the only investigator (apart from the present writer) who has taken titration readings in presence of formaldehyde at other points along the titration curve, in addition to the end-point. But his work, dealing with “the Formol Titration of Bacteriological Media” (7) is not concerned with the calculation of apparent p_k values, or with mass-law equations, or the like, nor do his readings show the effect of variations in formaldehyde concentration upon the titration curves, to which effect, however, he does make a passing allusion (*loc. cit.*, p. 249). His paper was published almost simultaneously with my first paper (3) in which I predicted the value of the apparent p_k constants for formol titrations (*loc. cit.* (3), p. 501). His careful readings are found to be admirably in keeping with my suppositions, and are employed below in conjunction with and in confirmation of my own more extended results.

No work appears to have been published hitherto on the HCl titration curves of amino-acids in presence of formaldehyde.

to proteins (1925, *loc. cit.* (4), *e.g.*, pp. 369, 379). Levene and Simms (6) (1925) were the first to give a mathematical formula connecting “G values for dibasic acids” (which are identical in magnitude with my titration dissociation constants) with the classical constants; and Simms has recently (1926) (6A) worked out in detail the mathematical relations existing between the various ionisation constants of a polyvalent substance, and has correlated these values with its chemical structure. Comparison must also be made with the “intrinsic” constants of E. Q. Adams, 1916 (6B) which relate to the removal of the respective hydrogen atoms in a poly-acid from the non-ionised molecule, and which he, likewise, connected with chemical constitution.

Experimental.

Method.—Consideration of the factors involved and of the special objects of the investigation determines various conditions and restrictions, under which the experimental readings have to be carried out :—

- (a) The concentration of formaldehyde should be kept constant for every reading on the titration curve, in the present section. (It is shown later, section II, that a titration reading at a given p_H can be duplicated only when the formaldehyde concentration is the same on each occasion.)
- (b) The concentration of amino-acid should be kept constant. (Effect of variation is considered in the second half of the paper.)
- (c) The titrant (NaOH) should be added in as highly concentrated a condition as convenient. (By this means the total volume of the titrated fluid is kept nearly constant, and thereby the approximate constancy of concentrations of amino-acid and formaldehyde is conserved. Theoretically, titration with a highly concentrated reagent is advantageous (8).)
- (d) The p_H should be determined colorimetrically (in order that the results may be applicable to the technique of the Sørensen method where the determination is made with a colorimetric indicator. The use of an electrometric method would permit of a higher absolute degree of accuracy, yet colorimetric results are sufficiently accurate for our present purpose).
- (e) Determinations of blank corrections for the solvent must be made (since we are concerned only with the titration curve of the amino-acid or its methylene derivative or both and not with that of the other constituents of the mixture; and this blank correction is considerable and highly variable on account of the high buffering capacity of the formaldehyde present).

The foregoing desiderata are fulfilled in the following technique.

Experimental Details.

Titration was made to match a series of pre-determined p_H virages shown by Clark and Lub's indicators in aqueous (non-formol) solutions.

Enough formalin was first pipetted out to yield the final formaldehyde concentration desired (*e.g.*, 8 per cent. in Table I; sometimes 16 per cent.), the appropriate volumes of water and indicator were next added, and N/1

soda was then run in from a micro-burette (graduated in 0.01 c.c.) (Standley, Belcher & Mason, Ltd.) until the tint accurately matched that shown in the standard buffer solution—which had the given p_H value, the same total volume, the same concentration of indicator, and was contained in a similar comparison vessel. This burette reading is the approximate blank for the amount of formalin taken; subsequent slight dilution caused by the addition of amino-acid and further soda causes a comparatively negligible increase in its magnitude. The amino-acid (e.g., 5.0 c.c. of M/10 glycine in Table I) was then added, and normal soda run in once more until the same tint was matched. This reading represents the corrected titre. (The amount of formaldehyde entering into chemical combination with the acid is very small compared with the total amount present and may be neglected in computing the formol blank.)

Readings with glycine are given in Table I.

Table I.—Titration of Glycine (0.025 m.) in presence of Formaldehyde, 8 per cent., *ca.*

(1)		(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Indicator and p_H virage.		Volume of formalin (40 per cent. formaldehyde) taken.	Volume of water taken.	Volume of indicator taken.	Volume of N/1 NaOH required in blank titration.	Volume of M/10 glycine added.	Volume of additional N/1 NaOH now required.	Final total volume.	Final concentration of formaldehyde.	Final concentration of amino-acid.
		c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	Per cent.	m.
P.B.B.	4.0	4	10	1.0	0.01	5.0	0.01	20.02	8.0	0.0250
M.R.	5.0	4	10	1.0	0.025	5.0	0.05	20.075	8.0	0.0249
M.R.	5.4	4	10	1.0	0.03	5.0	0.22	20.25	7.9	0.0247
B.C.P.	5.8	4	10	0.8	0.04	5.0	0.32	20.16	7.9	0.0248
B.T.B.	6.6	4	10	1.0	0.035	5.0	0.42	20.455	7.8	0.0244
P.R.	7.4	4	10	1.0	0.035	5.0	0.47	20.505	7.8	0.0244
T.B.	8.3	4	10	1.0	0.045	5.0	0.50	20.545	7.8	0.0243

Readings in col. 5 were sensitive to $\pm < 0.005$ c.c., and in col. 7 to ± 0.01 or 0.02 c.c.; p_H values of buffer solutions in col. 1 were accurate to $< 0.2 p_H$.

In fig. 1, values of p_H (indicator virage) (column 1) are plotted against corrected titres (column 7). It will be observed that all the readings lie accurately (within the experimental error) on the path of the titration curve for an acid (or base) having $p_K = 5.7$.

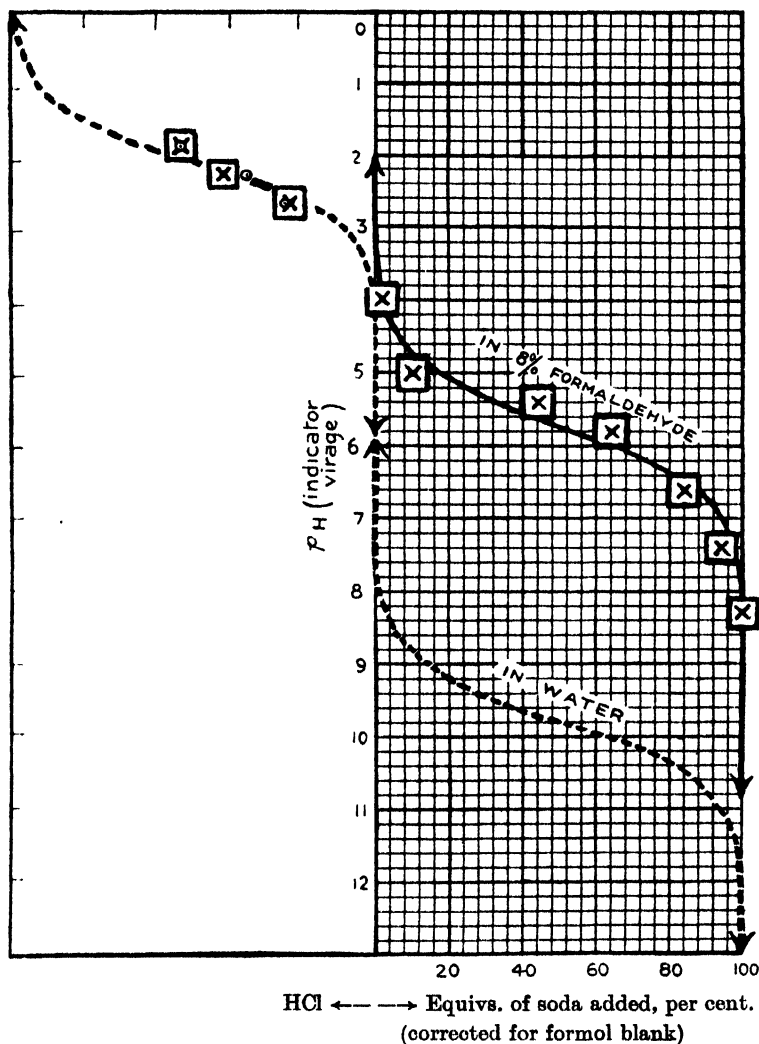


FIG. 1—TITRATION OF GLYCINE IN 8 PER CENT. FORMALDEHYDE (7.8—8.0 PER CENT.).

On the right—

- ⊗ Experimental titration readings (showing dimensions of possible experimental error in the technique employed).
- Henderson-Hasselbalch curve for dissociation constant $p_k = 5.7$.
- Titration curve of free glycine, corr. ($p_{k_a} = 9.75$).

(To the left is shown the titration with HCl, for which $p_{k_w} - p_{k_b} = 2.0$,* in water. Readings in formol lie on the same curve.)

* The indicator whose use is here shown gave a somewhat low value for p_{k_b} in water; the small circles represent the readings in water with HCl. In the extended work with NaOH concordant results were obtained from a series of different indicators.

In Table II the p_k values are calculated algebraically, as if one were dealing with a single acid, the methylene derivative of the amino-acid in question. (Values approaching the 0 per cent. and 100 per cent. axes, where the curve becomes asymptotic, are, of course, not employed.)

Table II.—Calculation of an Apparent p_k Constant for Titration of Glycine in presence of Formaldehyde, 8 per cent.

p_H virage.	α .	$p_k = p_H - \log \frac{\alpha}{1-\alpha}$.
5.0	0.1	5.95
5.4	0.44	5.50
5.8	0.64	5.55
6.6	0.84	5.88
		Average 5.7

Calculations for free glycine in Table III are from electrometric (and colorimetric) readings published earlier (1923) (1).

Table III.—Calculation of p_{k_a} for Titration of Free Glycine with NaOH.

p_H .	α .	$p_{k_a} = p_H - \log \frac{\alpha}{1-\alpha}$.
8.08	0.02	9.77
8.38	0.04	9.76
8.90	0.12	9.77
9.16	0.20	9.76
9.57	0.39	9.76
9.91	0.59	9.75
10.30	0.79	9.73
		Average 9.76

Similar methods were used for titrations with HCl. The readings are summarised in Tables IV and V. To economise space algebraic calculations of p_{k_b} values are omitted, results being shown graphically in fig. 1. More extended series of readings with HCl are now being taken.

Table IV.—Titration of Glycine (0.025 m.) with HCl, in 8 per cent. Formaldehyde.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
Indicator and p_H virage.	Volume of formalin (40 per cent. formaldehyde) taken.	Volume of water taken.	Volume of indicator taken.	Volume of M/10 glycine taken.	Volume of N/1 HCl required.	Blank correction, N/1 HCl, for same volume of formalin plus H_2O .	Volume of N/1 HCl, corrected.	Final total volume.	Final concentration of formaldehyde.	Final concentration of amino-acid.
	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	Per cent.	m.
T.B. 1.8	4.0	10.0	1.0	5.0	0.35	0.08	0.27	20.35	7.86	0.0246
T.B. 2.2	4.0	10.0	1.0	5.0	0.27	0.06	0.21	20.27	7.89	0.0247
T.B. 2.6	4.0	10.0	1.0	5.0	0.17	0.04	0.12	20.17	7.93	0.0248

Order of accuracy as in Table I.

Table V.—Titration of Glycine (0.025 m.) with HCl in Water.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Indicator and p_H virage.	Volume of water taken.	Volume of indicator taken.	Volume of M/10 glycine taken.	Volume of N/1 HCl required.	Blank correction, N/1 HCl, for same volume of water.	Volume of N/1 HCl, corrected.	Final total volume.	Final concentration of amino-acid.
	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	m.
T.B. 1.8	14.0	1.0	5.0	0.36	0.09	0.27	20.36	0.0246
T.B. 2.2	14.0	1.0	5.0	0.24	0.06	0.18	20.24	0.0247
T.B. 2.6	14.0	1.0	5.0	0.17	0.04	0.13	20.17	0.0248

Order of accuracy as in Table I.

Results.

Whereas the titration of free glycine with soda gives a curve having $p_k = 9.75$, titration in presence of 8 per cent. formaldehyde is seen to conform accurately with the curve for $p_k = 5.7$. That is to say, a ten-thousand-fold increase in the apparent acidity of glycine occurs upon the addition of formaldehyde (8 per cent.), titration of glycine in water and formaldehyde respectively

showing the following differences in end-point towards indicators (apparent p_H values) :

	In water.	In formaldehyde (8 per cent.).
Virtual beginning of titration with soda	7.75	3.7
Mid-point of titration	9.75	5.7
Virtual end-point of titration	11.75	7.7

The k_b curve, on the other hand, (titration with HCl) remains virtually unchanged.

Readings with other Amino-acids.

The following amino-acids have also been titrated with NaOH in a similar manner :-

Mono-amino Mono-carboxylic Acids.--Alanine, phenylalanine.

Hydroxy-amino Acid.—Tyrosine.

Mono-amino Di-carboxylic Acids.—Glutamic and aspartic acids.

The di-amino mono-carboxylic acids, lysine and arginine, are found to behave in a distinctive manner, which warrants their being considered in a separate paper.

To save space, tables of the experimental values are omitted, readings being plotted only ; see graphs, figs. 2 to 6. Whenever possible Brown's values (*loc. cit.*) have been redetermined and incorporated ; his "formol titration values" being corrected for the amino-acid blank (see below, p. 423), and all his figures recalculated to the basis of percentage equivalents of soda.

Experimental Details.

Experimental procedure was as for glycine unless otherwise specified :—*Formaldehyde concentration* was approximately 16 per cent. to conform with Brown's conditions (*i.e.*, varying from 17.8 per cent. to 14 per cent.). *Amino-acid concentrations* were approximately 0.005 m. (under Brown's conditions they are found to vary from 0.0044 m. to 0.0056 m.). *Final volume* of titrated liquid was about 20 c.c. (Brown, 18 c.c. to 23 c.c.). *Temperature* was 18° for titration in water (or else 25°, which gives only negligible divergence), and at room temperature for the titrations in formaldehyde. *Complete titration curves* are shown for amino-acids in water ; Brown's results extend to the first 50 per cent. of the titration only. Brown's titrations were carried

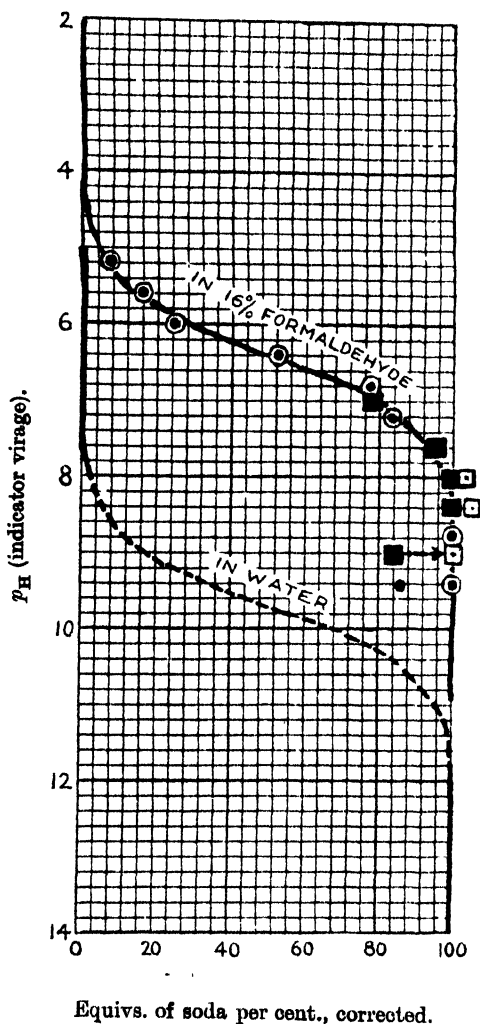


FIG. 2.—TITRATION OF ALANINE IN 16 PER CENT. *ca.* FORMALDEHYDE.

- Theoretical for $p_k = 6.4$.
- Experimental. Brown's formol titrations.
- Experimental. Latter corrected by present writer for amino-acid blank.
- Experimental. Brown's "resultant curve (4)," recalculated by present writer.
- - - Titration curve of free alanine ($p_{ka} = 9.7$).

out with N/20 soda, which involves a greater change in final *dilution* than those with N/1 soda recorded above. Brown's *formol blank* differs in having been done on a separate control specimen of formalin, instead of on the actual

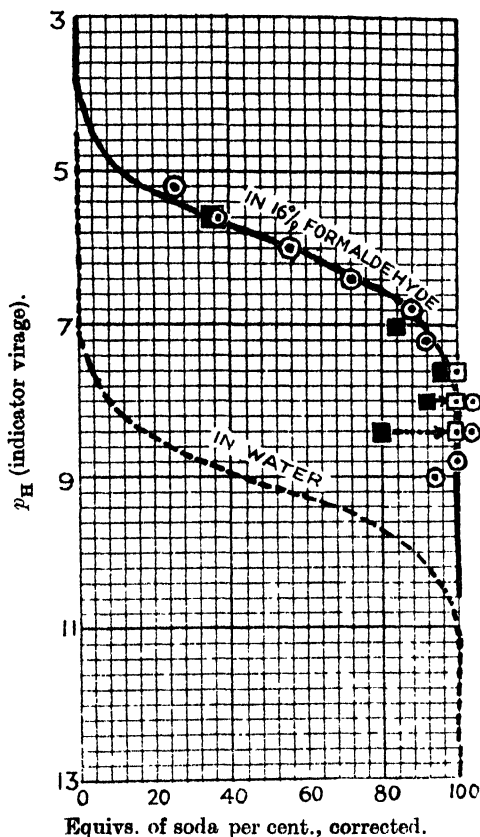


FIG. 3.—TITRATION OF PHENYLALANINE IN 16 PER CENT. *ca.* FORMALDEHYDE.

— Theoretical for $p_K = 5.9$.

} Experimental values as in fig. 2.

---- Titration curve of free phenylalanine ($p_{K_a} = 9.1$).

specimen subsequently added to the amino-acid for the titration: from a practical point of view the accuracy of his method is about as good.

Correction of Brown's formol titration readings for the amino-acid blank:— In Brown's "formol titration" the amino-acid was titrated with soda (in presence of formaldehyde already at the given p_H value), after the free amino-acid had itself been previously brought to this same p_H value by the addition of soda. To obtain true titration curves descriptive of the methylene derivatives,

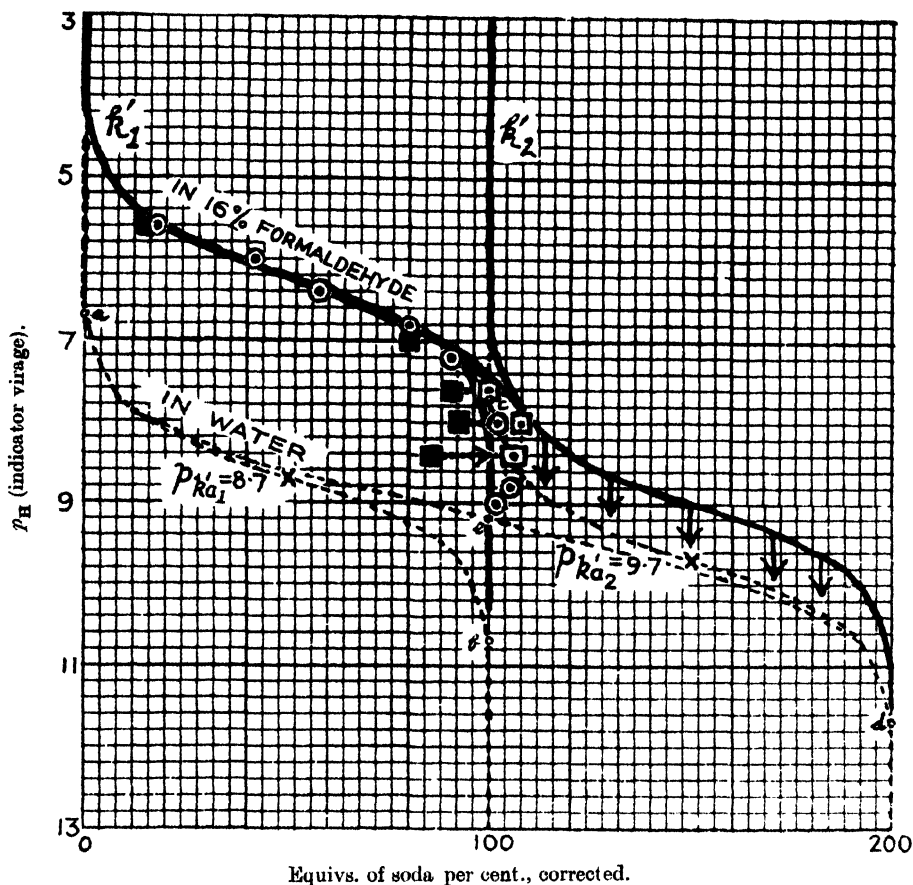


FIG. 4.—TITRATION OF TYROSINE IN 16 PER CENT. FORMALDEHYDE.

— Theoretical for $p_{K'a_1} = 6.2$, and $p_{K'a_2} > 9$.

■ }
 □ } Experimental values as in fig. 2.
 ○ }

---- Titration curve of free tyrosine,
 a b = curve for $p_{K'a_1} = 8.7$.
 c d = curve for $p_{K'a_2} = 9.7$.

titration dissociation constants, p. 414.

a e d = resultant experimental curve, obtained by adding the abscissæ at each point in the above separate curves.

(Slightly varying values for the dissociation constants of tyrosine in water have been obtained under somewhat varying circumstances:—(1) L. J. Harris, 1923 (1), p. 473; and P. Hirsch, 1924 (9); cf. Hitchcock for intermediate values, $p_{K'a_1} = 9.11$, $p_{K'a_2} = 10.07$. The above experimental curve gives values similar to Hirsch's.)

I have corrected Brown's formol titration readings at each p_H value by adding thereto the amounts of soda calculated to be necessary to bring the free amino-acid itself to the same p_H value. The corrected readings thus obtained are found to agree closely with Brown's "resultant curve, 4."

The titration curves for the amino-acids *in presence of water* are taken from the experimental readings previously published (1923 (1), p. 470, *et seq.*).

Calculation of Apparent p_k Values.

As for glycine, p_k constants have been calculated by the (slightly modified) Henderson-Hasselbalch equation, p. 413.

Table VI.—Calculation of Apparent p_k Constants for Titration of various Amino-Acids (0.005 m.) in presence of 16 per cent. Formaldehyde.

p_H .	Equivalents of soda added to amino-acid, per cent.	$p_k = p_H - \log \frac{a}{1-a}$.
<i>Alanine.</i>		
5.2	8	6.35
5.6	16	6.5
6.0	24	6.375
6.4	52	6.3
6.8	76	6.45
7.2	84	6.4
		Average 6.4
<i>Phenylalanine.</i>		
5.6	38	5.81
6.0	56	5.9
6.4	72	5.99
6.8	88	5.9
		Average 5.9
<i>Tyrosine.</i>		
5.6	17	6.3
6.0	42	6.125
6.4	58	6.25
6.8	80	6.2
7.2	90	6.25
		Average 6.2
<i>Aspartic Acid.</i>		
6.0	12	6.9
6.4	24	6.9
6.8	48	6.85
7.2	68	6.85
7.6	88	6.7
		Average 6.85

Table VI—(continued).

p_H	Equivalents of soda added to amino-acid, per cent.	$p_K = p_H - \log \frac{a}{1-a}$
<i>Glutamic Acid.</i>		
6.0	14	6.8
6.4	28	6.8
6.8	52	6.775
7.2	70	6.725
7.6	88	6.7
		Average 6.77

Findings as to p_K constants are summarised below.

Table VII.—Comparison of Apparent Dissociation Constants of Amino-Acids in Aqueous and in Formaldehyde Solutions (Methylene Derivatives).

	In water (free amino-acid).	In formaldehyde (ca. 16 per cent.) (methylene derivative).	Order of increase $\frac{k_{\text{in formaldehyde}}}{k_{\text{in water}}}$
	p_{K_a}	$p_{K'_a}$	
Glycine	9.75	5.4	10^4
Alanine.....	9.7	6.4	10^3
Phenylalanine	9.1	5.9	10^3
Tyrosine	8.7	6.2	10^3
Tyrosine, $p_{K'_a_2}$	9.7	>9	—
Aspartic acid	3.8	≈ 3.8	—
Aspartic acid, $p_{K'_a_2}$	9.85	6.85	10^3
Glutamic acid	4.2	≈ 4.2	—
Glutamic acid, $p_{K'_a_2}$	9.8	6.8	10^3

Results.

The titration curve of each amino-acid with NaOH in presence of formaldehyde (16 per cent., *ca.*) is seen to conform closely with the theoretical curve for a body having an apparent acidic ionisation constant 1000-times as great as that of the free amino-acid itself in aqueous solution. (In the case of one amino-acid, glycine, the increase is somewhat greater still.)

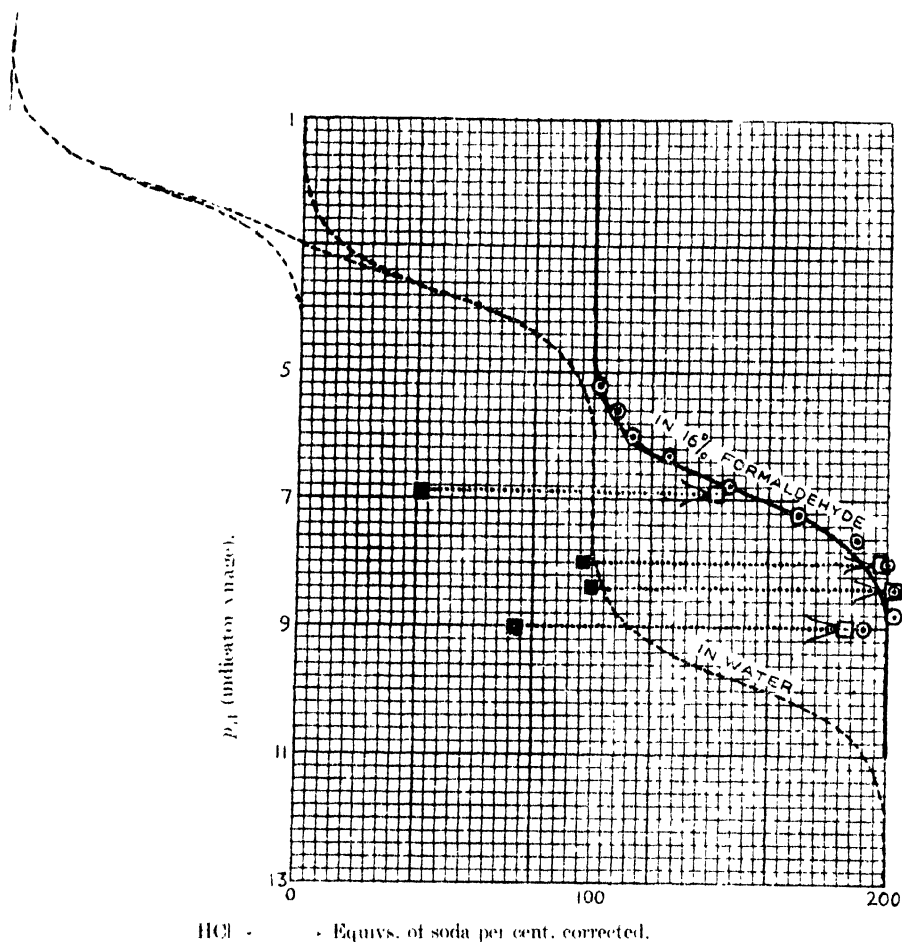
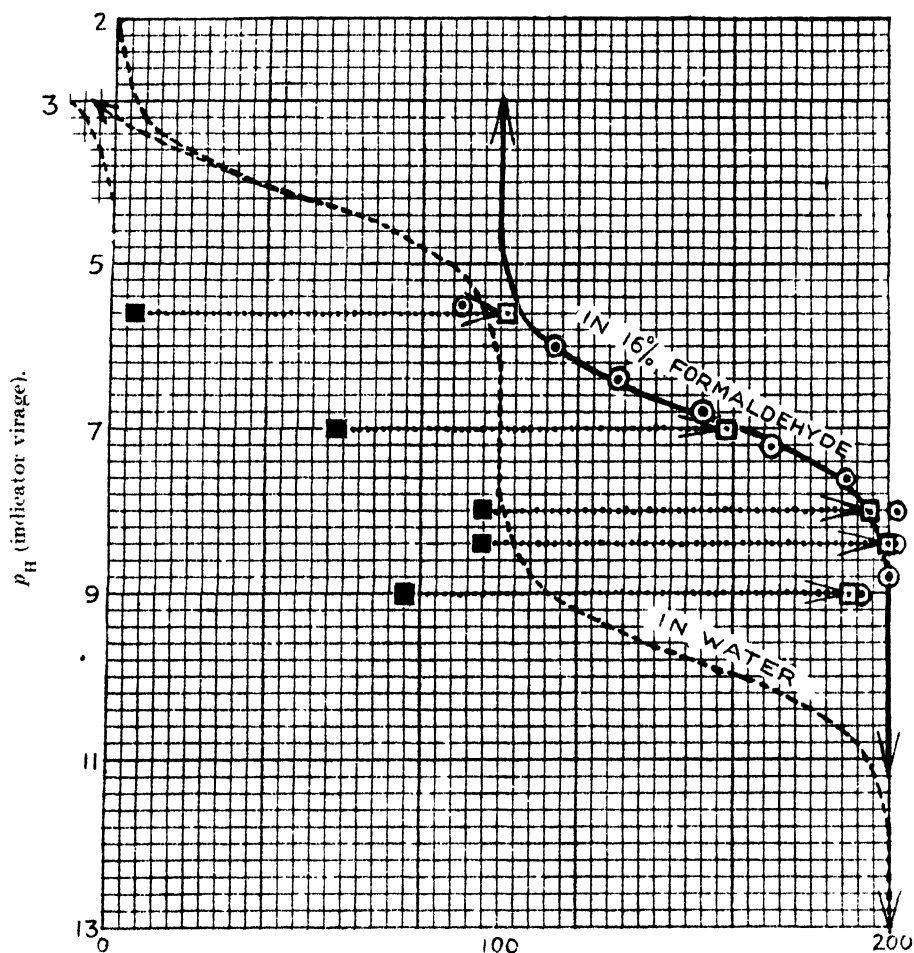


FIG. 5.—TITRATION OF ASPARTIC ACID IN 16 PER CENT. *ca.* FORMALDEHYDE.

— Theoretical for $p_{K_2} = 6.85$
 ■ } Experimental values as in fig. 2.
 [] }
 ○ }
 - - - Titration curve of free aspartic acid ($p_{K_{a_1}} = 3.8$, $p_{K_{a_2}} = 9.85$. The titration with HCl is also shown, $p_k = p_{K_{a_1}} - p_{K_b} = 2.1$).



HCl <--- ---> Equivs. of NaOH per cent. corrected.

FIG. 6—TITRATION OF GLUTAMIC ACID IN 16 PER CENT. *ca.* FORMALDEHYDE

— Theoretical for $p_{k'_2} = 6.8$.

■ }
 □ } Experimental values as in fig. 2
 ○ }

--- Titration curve of free glutamic acid, $p_{k'_{a_1}} = 4.2$.

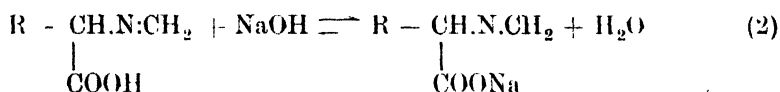
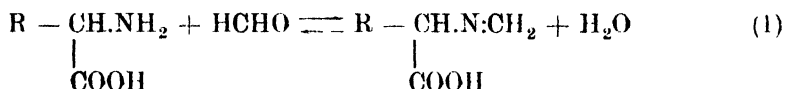
$p_{k'_{a_2}} = 9.8$.

$(p_{k_w} - p_{k_b} = 2.2)$.

II.—EFFECT OF VARYING THE FORMALDEHYDE CONCENTRATION.

No record can be found in the literature of any investigation having been made of the titration curves of amino-acids in the presence of varying concentrations of formaldehyde.

SØRENSEN (*loc. cit.*) noted that low results were obtained in his titrations to the end-point of p_{H} 9.0 when the formaldehyde concentration fell below about 12 per cent. This result he attributed to the reaction (1) being forced from right to left in presence of insufficient formaldehyde :-



BROWN (*loc. cit.*) stated similarly that it was unnecessary to have a formaldehyde concentration of more than about 16 per cent. for the purposes of a formol estimation as carried out according to his specifications.

Other workers have used widely divergent quantities, in some cases with erroneous results. Kendall, Day and Walker (11) took a formaldehyde concentration of only 4 per cent., while in Bermann and Rettger's (12) investigations it was as low as 1.5 per cent. and under.

Experimental.

Procedure.—The method adopted was similar to that described on p. 416, except that the various complete titration curves were each investigated at a different constant concentration of formol, *e.g.*, 0.5 per cent. to 32 per cent. formaldehyde in Tables VIII to X.

Table VIII.—Titration of Glycine (0.025 m.) in 0.5 per cent. Formaldehyde.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Indicator and p_H virage.	Volume of formalin (40 per cent. formaldehyde) taken (c).	Volume of water taken.	Volume of indicator taken.	Volume of N/1 NaOH required in blank titration.	Volume of M/10 glycine added.	Volume of additional N/1 NaOH now required.	Final total volume.	Final concentration of formaldehyde.	Final concentration of amino-acid.
	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	Per cent.	m.
T.B. 2.2	0.25	13.75	1.0	(-) 0.04 (a)	5.0	(-) 0.2(a) (b)	20.24	0.49	0.025
M.R. 5.4	0.25	13.75	1.0	—	5.0	0.0	20.00	0.50	0.025
B.T.B. 6.2	0.25	13.75	1.0	—	5.0	0.015	20.015	0.50	0.025
B.T.B. 6.6	0.25	14.75	1.0	—	5.0	0.04	21.04	0.48	0.024
P.R. 7.4	0.25	14.25	0.5	—	5.0	0.20	20.20	0.50	0.025
C.R. 7.8	0.25	13.75	1.0	—	5.0	0.23	20.23	0.49	0.025
T.B. 8.8	0.25	13.75	1.0	0.005	5.0	0.43	20.435	0.49	0.024

Effect of varying the Amino-Acid Concentration (from 0.005 to 0.075 m.).

											Titre a.
C.R.	7.8	0.25	17.75	1.0	—	1.0	0.4	20.4	0.49	0.005	0.4
C.R.	7.8	0.25	17.75	1.0	—	2.0	0.8	21.8	0.46	0.009	0.4
C.R.	7.8	0.25	13.75	1.0	—	5.0	0.23	20.23	0.49	0.025	0.46
C.R.	7.8	0.255	—	1.0	—	15.0	0.82	17.075	0.60	0.088	0.54

*** Note that the titre for a given p_H remains approximately constant (within the experimental error) irrespective of changes in amino-acid concentration. (Experimental accuracy as in Table I.)

(a) N/1 HCl required instead of N/1 NaOH.

(b) Corrected for separate blank.

(c) Added from micro-burette graduated in 0.01 c.c.

Table IX.—Titration of Glycine (0.025 m.) in 2 per cent. Formaldehyde.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Indicator and p_H virage.	Volume of formalin (40 per cent. formaldehyde) taken.	Volume of water taken.	Volume of indicator taken.	Volume of N/1 NaOH required in blank titration.	Volume of M/10 glycine added.	Volume of additional N/1 NaOH now required.	Final total volume.	Final concentration of formaldehyde.	Final concentration of amino-acid.
	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	Per cent.	m.
B.P.B. 3.4	1.0	13.0	1.0	(—) 0.005(a)	5.0	() 0.01(a)	20.015	2.00	0.025
M.R. 5.0	1.0	13.0	1.0	0.005	5.0	0.0	20.005	2.00	0.025
M.R. 5.4	1.0	13.0	1.0	0.005	5.0	0.01	20.045	2.00	0.025
B.C.P. 5.8	1.0	13.2	0.8	0.01	5.0	0.11	20.12	1.99	0.025
B.T.B. 6.2	1.0	13.0	1.0	0.01	5.0	0.15	20.16	1.98	0.025
B.T.B. 6.6	1.0	13.0	1.0	0.01	5.0	0.22	20.23	1.98	0.025
P.R. 7.4	1.0	13.5	0.5	0.01	5.0	0.36	20.37	1.96	0.025
C.R. 7.8	1.0	13.0	1.0	0.01	5.0	0.42	20.43	1.96	0.025
T.B. 8.8	1.0	13.0	1.0	0.015	5.0	0.49	20.505	1.95	0.024

Effect of varying the Amino-Acid Concentration (from 0.01 to 0.075 m.).

										Titre = a.
B.T.B. 6.6	1.0	16.0	1.0	0.01	2.0	0.075	20.085	1.99	0.010	0.4
B.T.B. 6.6	1.0	13.0	1.0	0.01	5.0	0.22	20.23	1.98	0.025	0.44
B.T.B. 6.6	1.02	3.0	1.0	0.01	15.0	0.72	20.75	1.97	0.072	0.48

*** Note that the titre for a given p_H remains approximately constant (within the experimental error) irrespective of changes in amino-acid concentration. (Experimental accuracy as in Table I.)

(a) N/1 HCl required instead of N/1 NaOH.

Table X.—Titration of Glycine (0.025 m.) in 32 per cent. Formaldehyde.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Indicator and p_H virage.	Volume of formalin (40 per cent. formaldehyde) taken.	Volume of water taken.	Volume of indicator taken.	Volume of N/1 NaOH required in blank titration.	Volume of M/4 glycine added.	Volume of additional N/1 NaOH now required.	Final total volume.	Final concentration of formaldehyde.	Final concentration of amino-acid.
	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	Per cent.	m.
T.B. 2.2	16.0	1.0	1.0	(-) 0.05(a)	2.0	(-) 0.15(a)	20.20	31.7	0.025
B.P.B. 3.4	16.0	1.0	1.0	0.03	2.0	0.0	20.03	32.0	0.025
B.P.B. 4.0	16.0	1.5	0.5	0.07	2.0	0.08	20.15	31.8	0.025
B.C.G. 4.4	16.0	1.0	1.0	0.10	2.0	0.15	20.25	31.6	0.025
M.R. 5.0	16.0	1.0	1.0	0.12	2.0	0.31	20.43	31.3	0.024
M.R. 5.4	16.0	1.0	1.0	0.13	2.0	0.455	20.585	31.1	0.024
B.C.P. 5.8	16.0	1.2	0.8	0.13	2.0	0.495	20.625	31.0	0.024
T.B. 8.8	16.0	1.0	1.0	0.36	2.0	0.50	20.86	30.7	0.024

(a) N/1 HCl required instead of N/1 NaOH.

In further experiments series of titrations were carried out all to some pre-determined constant value of p_{H_2} and the effects were observed of changes in the formaldehyde and amino-acid concentrations upon the experimental titre (see Table XI, p. 434).

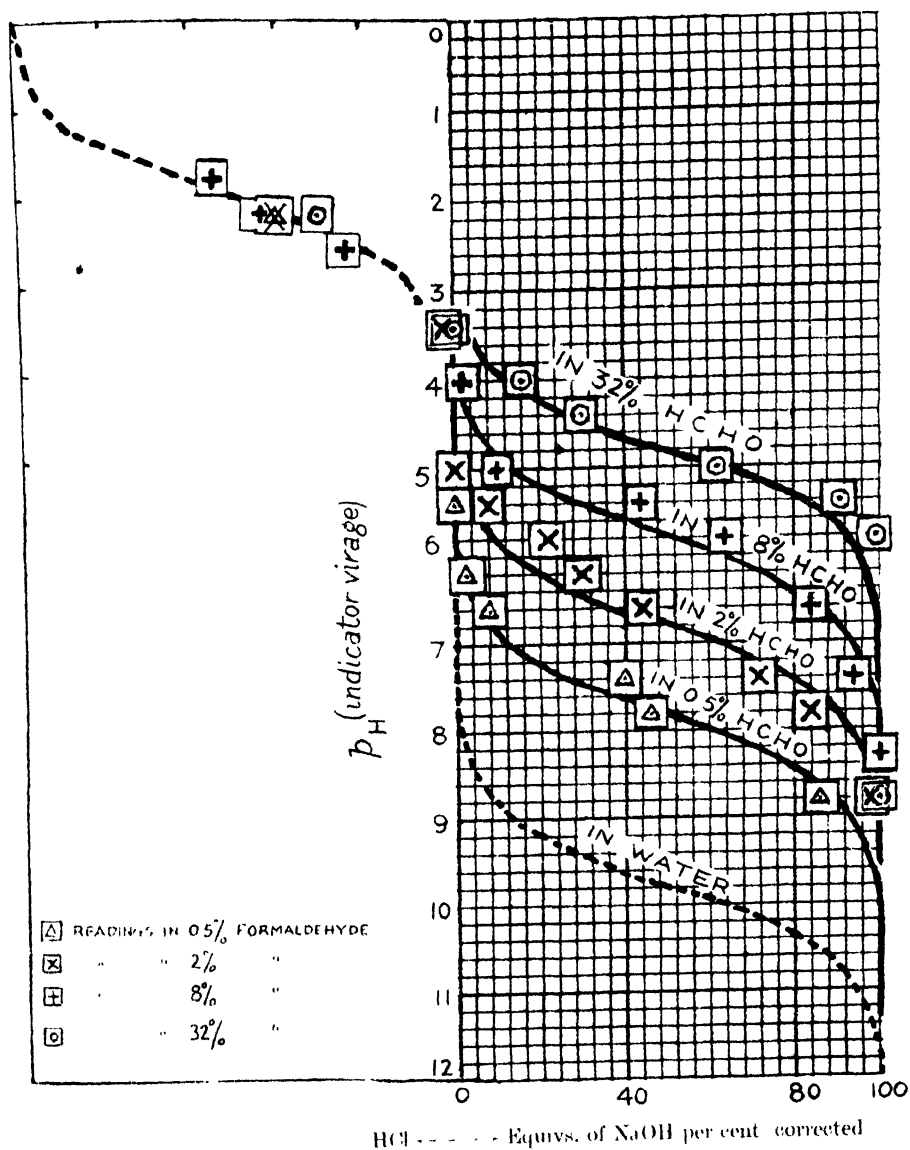


FIG. 7.—EFFECT OF VARIATIONS IN FORMALDEHYDE CONCENTRATION UPON THE TITRATION CURVE OF GLYCINE.

Each fourfold increase in formaldehyde concentration is seen to result in unit decrease in $p_{K'_a}$ (= a tenfold increase in K'_a), i.e. in a tenfold increase in the H ion concentration, which results after a given addition of NaOH. The titration with HCl, on the other hand, remains practically unchanged by increase in formaldehyde concentration.

Table XI. - Variation in Amounts of Soda required in Titrating Glycine to p_H 5.4, in presence of varying Concentrations of Formaldehyde. (Note that the p_K value remains the same for a constant $[HCHO]$, irrespective of $[amino-acid]$ or of $[HCHO]:[amino-acid]$).

Volume of formalin.	Strength of formalin.	Volume of M 10 amino-acid.	Volume of N/10 NaOH (correction for blanks).	Total volume.*	Concentration of formaldehyde	Amino-acid.	$Title = a = \frac{[NaOH]}{[Glycine]}$	$\log \frac{a}{1-a}$	$p_H = p_H - \log \frac{a}{1-a}$
c.c.	Per cent.	c.c.	c.c.	c.c.	Per cent.	m.			
40	20	5	3.6	52.65	15.2	0.0095	0.72	0.4	5.0
10	20	5	3.75	52.80	15.2	0.0095	0.75	0.5	4.9
10	20	5	2.45	18.7	10.7	0.0267	0.59	0.2	5.2
10	20	5	0.75	50.4	4.0	0.0099	0.15	-0.75	6.2
10	20	5	3.6	52.65	15.2	0.0095	0.72	0.4	5.0
80	20	5	3.7	96.9	16.5	0.0052	0.74	0.5	4.9
80	40	5	4.9	102.6	31.22	0.0049	0.98	1.7	3.7
N/1 NaOH.									
10	40	5	0.4	21.52	18.6	0.0233	0.80	0.6	4.8
10	40	10	0.79	21.875	18.3	0.0457	0.79	0.6	4.8
10	40	2	0.16	21.24	18.8	0.0094	0.80	0.6	4.8

* For brevity, the varying amounts of water and indicator added are omitted from the table.

In fig. 8 *complete* titration curves in presence of varying concentrations of formaldehyde are reconstructed from the *single* readings shown in Table IX.

Results.

1. The amount of soda necessary to titrate the amino-acid to a given p_H value depends on the formaldehyde concentration and not upon the ratio of the reactants $[formaldehyde]:[amino\ acid]$ (equation (1) above) under the conditions examined (*e.g.*, formaldehyde 18 per cent. and amino-acid 0.009 m. to 0.04 m. at foot of Table XI). (The molar concentration of HCHO is immense compared with that of the amino-acid.)

2. For each formaldehyde concentration the titration curve conforms (within the experimental error) throughout its length with the titration curve of a mono-acid (or back titration curve of a mono-base) as defined by the Henderson-Hasselbalch equation (see fig. 7).

3. The apparent p_k value, defining this titration curve, diminishes (*i.e.*, acidity increases) with increasing formaldehyde concentration, a four-fold

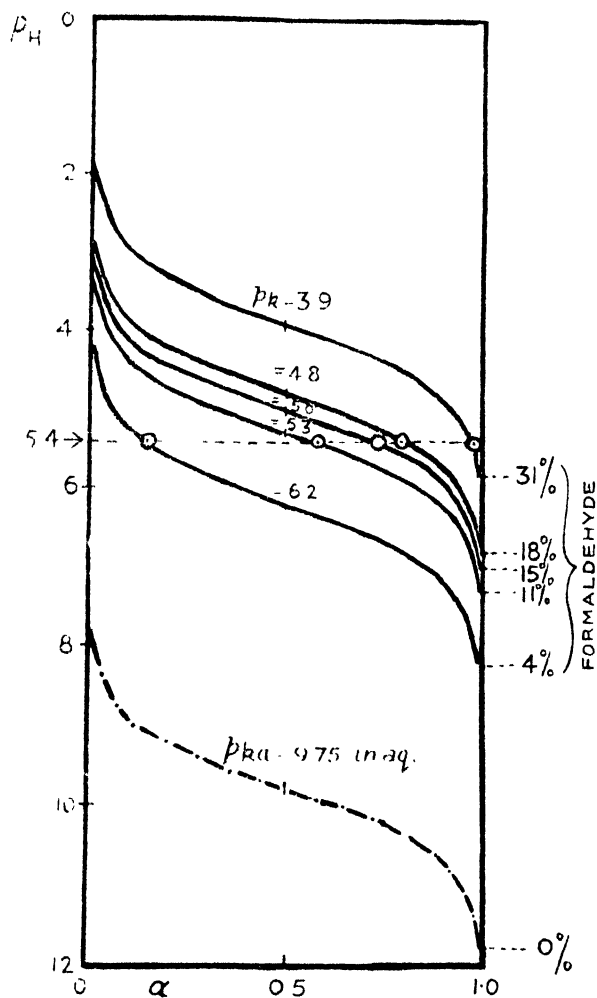


FIG. 8.—TITRATION CURVES OF GLYCINE TAKEN IN VARIOUS CONCENTRATIONS OF HCHO , EACH RE-CONSTRUCTED FROM A SINGLE READING TAKEN AT $p_{\text{H}} 5.4$ (r. Table XI).

(The accuracy of the reconstructed curve diminishes for readings of α approaching 0.0 or 1.0, where the curve is asymptotic.)

increase of formaldehyde concentration resulting in one unit increase of p_k . The present data are not intended to form the basis for highly accurate measurements, but the relation between p_k and [formaldehyde] is shown in a roughly

quantitative manner in fig. 9. Such data serve for calculating the various reaction constants involved.

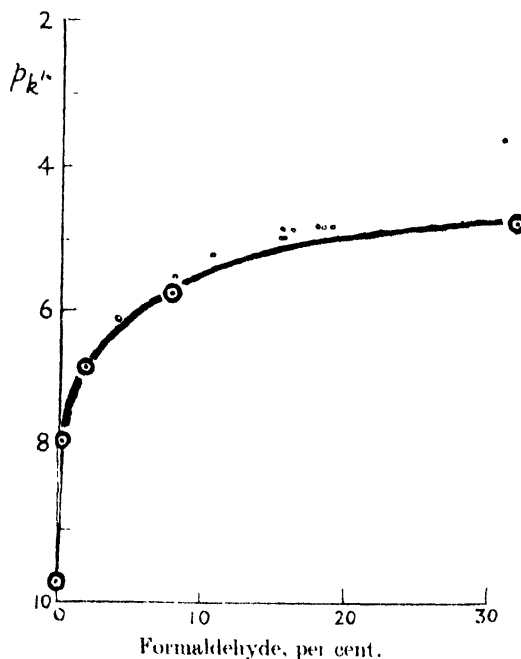
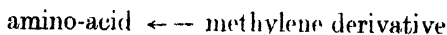


FIG. 9.—EFFECT OF FORMALDEHYDE CONCENTRATION upon the “apparent acidic” dissociation constant of GLYCINE.

- Calculated from single titration reading; $p_H = 5.4$.
- Calculated from complete titration curves.

1. The NaOH titration curves shown in fig. 7 do not in themselves bear evidence of the reaction



(which Sørensen believed to occur in the presence of insufficient alkali) in the sense of there being any deformation of that part of the experimental titration curve lying near the p_H axis, away from the theoretical Henderson-Hasselbalch curve and towards the curve for the free amino-acid.

5. The apparent basic strength of the ampholyte (titration with HCl) remains, however, virtually unaltered by addition of formaldehyde.

COMMENTS.

The aim of the present paper is to be descriptive of the experimental behaviour towards p_H indicators of the various amino-acids in presence of different concentrations of alkali and formaldehyde, rather than to analyse the theoretical

basis of the phenomena. It may be noted, however, that the equilibria concerned are involved, and that no strict physico-chemical meaning can be attached to the observed apparent p_k values without a consideration of the action of such diverse factors as : the effect of the high formaldehyde concentrations upon the p_H virage of the indicators themselves ; the various activity coefficients ; or, the dielectric constant of the aqueous formaldehyde medium. It is interesting to note in this connection that the observed behaviour of amino-acids towards indicators in presence of formaldehyde was earlier shown (*loc. cit.* (3), p. 501, etc.) to be paralleled by their behaviour in presence of alcohol : and that in the latter case we now know that the change in dielectric constant from water to alcohol plays an important part (13).

The Zwitterion Constitution of the Amino-acid Molecule.---The observed titration curve of glycine with soda *in water* may represent equally either (1) combination between COOH and Na^+ , or (2) replacement of the ionised $\text{R} \cdot \text{NH}_3^+$ by Na^+ , the p_k value observed relating in these two alternatives either (1) to the old ("apparent acid") constant or (2) to the new basic (zwitterion) constant (*loc. cit.* (4), p. 383). Likewise the HCl titration curve in water affords no indication *per se* of the nature of the chemical change, *i.e.*, whether the basic group is being directly titrated with the HCl or the acidic group being replaced (back titrated). Light is, however, thrown on the issue by the effect which we have found to be produced on the curves by the addition of formaldehyde. Now the express purpose of the formaldehyde added is to combine with the basic group of the amino-acid. Yet the results show that it is the *apparent* basic constant itself which remains unchanged and the *apparent* acidic constant which increases. Such an *apparently* anomalous effect supports the "zwitterion" theory that the *apparent* basic and acidic constants respectively relate in actuality to the *true* acidic and basic constants. This point of view leads to the more intelligible interpretation of the present results that the addition of formaldehyde causes a diminution of the *true* (zwitterion) basic constant and no significant change in the *true* acidic constant. This conclusion is confirmed by further work now in progress in which it is found that the addition of formaldehyde to an ammonium salt (such as $\text{Am}^+ \text{Ac}^-$) results also in a diminution of the *true* basic constant, whose value is here definitely known, the effect paralleling that already described for glycine. Other classes of amino-acids are being examined to decide into which category they fall.

Optimum Conditions for Formol Titration.---A scrutiny of the experimental results in water and formol will confirm the conclusion of J. H. Brown (*loc.*

cit.) that accurate results are obtainable under the following conditions, viz., the sample to be analysed is first brought to p_H 8 virage with phenol red, formaldehyde neutralised also to p_H 8 is next added, and the amino-acid content is then estimated by titration to the same p_H virage, the final concentration of formaldehyde being about 16 per cent. Some of the alternative methods of other writers already alluded to, will be found to result in less accurate, sometimes highly inaccurate, titres.

It should be added, however, that the writer considers his alcohol titration method (3) generally preferable to a formol titration. Martens (14) who recently submitted it to a critical examination (and applied it also to polypeptides) reported that he found it "the most accurate and convenient method available, being easier of execution than that of Foreman [Willstätter], and having two marked advantages over Sørensen's formol method, (1) ease of determining the end-point, and (2) precision of the results." This method in addition permits one to determine amino groups as part of the same titration.*

SUMMARY.

1. Curves are given showing the variation in p_H value (determined colorimetrically) with the amount of soda added, when various amino-acids are titrated in aqueous formaldehyde, each addition of soda being corrected for the acidity of the "solvent."

2. For a constant concentration of formaldehyde the corrected curve for each amino-acid conforms throughout its length with the Henderson-Hasselbalch equation for a simple acid possessing a determinate p_K value.

3. Apparent dissociation constants are accordingly calculated, as though one were dealing with a simple acid substance, viz., "the methylene derivative" of the amino-acid in question.

4. In concentrations of formaldehyde such as are most usually employed in the Sørensen titration (*e.g.*, as recommended by Brown) the apparent p_K values are approximately three units less than for the corresponding free amino-acids in aqueous solution: the rationale of the method therefore lies in the formation of methylene derivatives in equilibrium with the amino-acid having resultant NaOH-titration curves and p_H end-points displaced three units to the less alkaline side of the p_H scale, compared with the amino-acids themselves.

* A second method which I proposed for estimating the $-\text{COOH}$ and $-\text{NH}_2$ is by titration in water against p_H indicators (3). Felix and Müller who have tested it extensively (15), while confirming the anomalous behaviour of arginine, consider that this alternative also offers special advantages for many purposes.

5. Within such limits of concentration as are common in a Sørensen estimation (*e.g.*, formaldehyde 2 per cent. to 18 per cent., amino-acid 0.005 to 0.05 *m.*) the apparent p_k value remained practically unaffected by the ratio of amino-acid to formaldehyde or by the concentration of amino-acid, when the concentration of formaldehyde was kept constant.

6. A progressive decrease in the apparent p_k values occurred on the other hand with increasing concentration of formaldehyde; the net effect therein resembling that of a change in the "medium," such as the addition of alcohol, where an alteration in the dielectric constant is involved.

7. The "apparent *basic*" constant of the amino-acid (determined by titrating with HCl) remained virtually unchanged by the addition of formaldehyde. This result together with the increase in the "apparent acidic" constant caused by the formaldehyde may be accounted for on the basis of the zwitterion hypothesis; according to which we have with the addition of formaldehyde no change in the *true* acidic constant (measured by titrating with HCl) but a diminution in the *true* basic constant (measured by titrating with NaOH).

I am indebted to Prof. R. A. Peters and Mr. J. B. S. Haldane for helpful criticism of this communication, and to Sir Frederick Hopkins, F.R.S., for his continual interest.

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Ionic Mobility as a Factor in Influencing the Distribution of Potassium in Living Matter.

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The investigation, the results of which are given in the following pages, was undertaken with the object of explaining, if possible, a number of anomalous findings in studies on the distribution of electrolytes, potassium more especially, in living matter.

The author, in his researches, begun nearly a quarter of a century ago and continued at times since then, on the localisation of potassium in animal and vegetable cells, by microchemical methods, found that this element is generally confined to interfaces in the cells, that is, everywhere where the tension is lower than it is in the rest of the cytoplasm. In *Acineta tuberosa* no potassium occurs in the cytoplasm generally, but traces of it obtain at the cytoplasm-spherule interfaces, and it is markedly present in the superficial membrane of each tentacle, the production and maintenance of which are due to a surface tension lower than that which obtains elsewhere on the surface of this unicellular protist (1913). In the secreting cells of the salivary glands and of the pancreas, and in the epithelial cells of the proximal convoluted tubules of the kidney in the dog, cat and frog, the potassium salts are confined to the luminal surfaces of the same, where it must be assumed the surface tension is low. In the cytoplasm of nerve cells no potassium obtains, but it is present in demonstrable quantities immediately under the surface of each axon, the production and maintenance of which are due to a surface tension lower than that which obtains on the general surface of the nerve cell. In striated muscle fibrils the potassium present is localised in the parts of the sarcous elements where the surface tension is presumably low.

In vegetable cells potassium is localised at interfaces where the tension is lower than it is elsewhere in such cells. This is particularly the case with the Protophyta (*Spirogyra*, *Mesocarpus*, *Cladophora*, *Oedogonium*, *Ulothrix* and *Zygnema*) (1912). In the cells of the higher plants (Metaphyta) the potassium present is not uniformly distributed throughout the cytoplasm but localised, either at points on the periphery where the surface tension must, judging from

the contour, be lower than elsewhere on the cell surface, or at cytoplasm-vacuole interfaces, in such a fashion as to suggest that such localisations are due to adsorption.

If all these localisations are due to adsorption, then according to the current conception regarding the causation of adsorption some potassium should be found diffusely distributed in the cytoplasm where an interfacial adsorption obtains as described. Such diffuse distribution has only rarely been found. This cannot be due to a failure of the method to demonstrate its presence in the cytoplasm, for the reagent used in the method (cobalt sodium hexanitrite), when freshly prepared, immediately precipitates potassium in water in which its concentration is as low as 1 in 500,000, as the author has found.

This at once raises the question whether the localisation of the potassium as indicated is in whole due to adsorption. If the localisation is not wholly due to adsorption, one must postulate that there are constituents of the interfaces which have such an affinity for, or capacity for combining with, potassium that all of the element is withdrawn from the cytoplasm and confined to such interfaces. The composition of the material forming the interfaces, one would infer from their causation, must at least be slightly different from that of the adjacent cytoplasm, that is, it must have elements or complexes which are wholly wanting or much less abundant in the general cytoplasm. The presence of such elements or complexes cannot, however, be demonstrated. A good case might be made out for them if sodium, which is less abundant than potassium in the cytoplasm, were wholly or almost wholly absent from the interfaces. That, however, has not been directly shown, for there is no method of demonstrating sodium microchemically in the cell.

Failing, therefore, to get any evidence that the cytoplasm of the interfaces has a special chemical affinity for potassium, one is led to inquire whether, after all, there are not forces or factors concerned in adsorption which, operating in the cytoplasm, would concentrate all its potassium at the interfaces where it is found, and which might perhaps account in some measure for the preponderance of this element as compared with sodium in the cell.

There is, indeed, one factor to the effect of which in adsorption not much attention has been given. This is ionic mobility, or ionic conductivity, the rate of transport to the anode and cathode of the ions of an electrolyte when a current of electricity is passed through a solution of it. The mobility of the ions of the different elements varies, those of hydrogen having the greatest, while those of lithium have the least, in the range of the monovalent elements. The mobility of potassium is nearly twice as great as that of lithium (65·3:

35.5), and nearly half as much again as that of sodium ($65.3 : 44.4$) at $18^{\circ}\text{C}.$ * These differences are all explained (Kohlrausch, Bousfield) as caused by the different degrees of hydration that these ions undergo when molecules containing these elements are dissociated in water, each lithium ion acquiring and retaining an envelope of water molecules thicker than that of each sodium ion, and each sodium ion an envelope thicker than that of each potassium ion - in other words the viscosity is greater with a lithium ion than it is with a sodium ion and with a sodium ion greater than it is with a potassium ion. The "mean free path" of the latter must, therefore, be greater than that of a lithium ion or a sodium ion. The electrostatic attraction between these ions and the anions with which they are associated in solutions modifies the mobility of the cations and the "mean free path" is affected, but if these cations are associated with a common anion, for example, that of chlorine, which has a mobility very slightly greater ($65.9 : 65.3$) than that of a potassium ion, the "mean free path" of the electrostatically associated potassium and chlorine ions must be greater than the "free path" of the electrostatically associated sodium and chlorine ions.

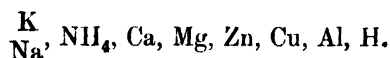
This greater ionic mobility of potassium ions as compared with that of sodium ions must have an influence on the rate and degree of adsorption of both kinds of ions. When in a solution of chlorides of these two elements new interfaces are developed, the ions which reach each interface first would, one may suppose, tend to cover it, thus excluding from it the less rapidly moving ions. This would involve, if the ions of both elements are present in equal numbers, an adsorption of those of potassium alone, or if those of sodium are present in excess of those of potassium, an adsorption of the latter in excess of their proportion to those of sodium.

To ascertain whether ionic mobility does so affect adsorption, nothing has been attempted, so far as a search in the literature of the subject would indicate. Determinations of the adsorption on blood charcoal of a number of electrolytes have been made by Lachs and Michaelis, Rona and Michaelis, Hartleben and others. These indicate that the cations and anions of an electrolyte are equally adsorbed, the amount adsorbed, however, depending on the electrolyte used. Only in three cases was a solution of two electrolytes used, each with chlorine as the anion (Rona and Michaelis). With $0.1\text{ N AlCl}_3 + 0.1\text{ N NaCl}$ the aluminium adsorbed was practically equal in amount to what was adsorbed from 0.2 N AlCl_3 alone. No estimation was made of the amount of sodium adsorbed. When a quantity of $0.1\text{ N AlCl}_3 + 0.1\text{ N HCl}$ was used no alu-

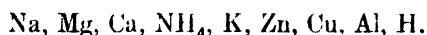
* Nernst, 'Theoretical Chemistry,' p. 408 (1923).

minium was adsorbed, and when a quantity of $0.1 \text{ N CuCl}_2 + 0.1 \text{ N HCl}$ was similarly used a much smaller amount of copper was adsorbed than was the case when 0.1 N CuCl_2 alone was used. The H ions thus appear to displace the Al ions completely, and the Cu ions very considerably. As the mobility of the H ion greatly exceeds that of any other ion, while that of the Na ion is lower than that of any other cation, lithium alone excepted, the suggestion occurs that ionic mobility was a factor in the production of these results.

According to Rona and Michaelis' determinations the cations from solutions of single electrolytes were adsorbed by charcoal in the following order of increasing concentration :—



If we arrange these in the order of the increasing mobility of their ions, it would be :—



This is not consistent with the order of adsorption ascertained by Rona and Michaelis and, consequently, though there is a certain amount of parallelism between the two series, it is not sufficient to justify accounting thereby for the order of the concentrations of the ions adsorbed on the charcoal. One must, therefore, to solve the question whether ionic mobility affects the adsorbability relatively of sodium and potassium from their salts in living matter, resort to direct experimental investigation of the subject to obtain results decisive, if at all possible, in the solution of it. This cannot be done, using living matter as an adsorbent, as may readily be understood, but with an adsorbent more or less inert chemically, which one can control, and thus maintain uniform throughout the investigation. This the author has attempted to do and he now presents the results.

In the selection of the adsorbent used consideration was given to two factors which might adventitiously affect the results. One of these was the degree of purity of the adsorbent, and the other the charge on the particles of the adsorbent. It is indeed difficult to find an absolutely pure adsorbent that would be inert under the influence of the chemical forces concerned in the adsorption of electrolytes. Charcoal made from pure cane-sugar should, one would suppose, be a pure adsorbent; but, and this Michaelis and Rona also found, it is exceedingly difficult (in fact, almost impossible) to grind it to a sufficiently fine powder to serve properly as a suitable adsorbent. Animal charcoal, even Merck's "Carbo Medicinalis" purified, is not pure. Michaelis and Rona, who

used it in their determinations of adsorption, found that 1.005 grm. of it on incineration gave 0.0708 grm. of white ash (or 7.044 per cent.) of which 0.0575 grm. (or 5.72 per cent.) was insoluble in hot concentrated hydrochloric acid. Of the soluble part, 0.0133 grm. (= 1.324 per cent.), one-seventh, *i.e.*, 0.0019 grm., was calcium. Iron was not present. The insoluble part, they suggested, consisted of calcium silicate.

The author found that the ash in two quantities of Merck's "Blood Charcoal purified by acid" amounted to 5.586 and 5.617 per cent., and, accordingly, less than the ash as found by Michaelis and Rona; and the part in each soluble in hot concentrated hydrochloric acid was 1.072 and 1.046 per cent. of the charcoal in each case incinerated. The calcium in the combined soluble salts was 0.1485 per cent., or about one-seventh of the total soluble salts, the iron present amounted to 0.00781 per cent., magnesium to 0.00582 per cent., and the sodium and potassium to 0.0611 and 0.1456 per cent. respectively. Phosphoric acid as phosphates, and chlorine as chlorides were present, but the percentage proportions of these were not determined.

These results are not in accord with those obtained by Michaelis and Rona, but theirs may be explained as derived from too small a quantity (1.005 grm.) of the charcoal analysed, whereas the author used for his analyses 3.786 and 6.720 grm. of the charcoal specified, dried at 115° C. for 96 hours.

The total salts found by Michaelis and Rona exceeded those found by the author, but the proportion of these as soluble salts was very nearly the same (18.78 and 18.90 per cent.). The proportion of calcium was practically the same. They reported iron absent, but, as pointed out, the quantity of charcoal they used for analysis was too small to furnish even any quantitative indications of it, and beyond the calcium content they did not determine the composition of 71.22 per cent. of soluble salts. Had they done so, it is very probable that the animal charcoal used by them was not, if at all, different from that analysed by the author.

The charcoal, apart from the ash which it yields on incineration, is not pure carbon. Elroy G. Miller found that when it was heated to below red heat hydrochloric acid was driven off, at a higher temperature substances with strong odours of cyanogen compounds were formed and volatilised and, at a still higher temperature, the gases evolved were alkaline. The hydrochloric acid present, he holds, is present as adsorpt, tenaciously held, and derived doubtless from the acid used in preparing this charcoal.

In a brand of this charcoal, recently introduced by Merck, with an ash content of 2.4 per cent. and with a much lower nitrogen content, Miller found

sulphuric acid present instead of hydrochloric acid, as adsorpt, derived doubtless from the acid used in preparing this brand. With quantities of this charcoal, used as adsorbent, large quantities of sodium hydroxide were neutralised, with the concomitant appearance of sodium sulphate in the solution. Such charcoals, Miller found, adsorbed acids, but not bases or salts, a result quite contrary to what had been previously obtained with such charcoals. In consequence many of the data derived from investigations on the adsorption by such charcoals must be open to question.

The acids present in these charcoals cannot be readily extracted from them. Treatment with hot water fifteen times, Miller found, did not remove more than half of the hydrochloric acid adsorbed, even though in each extraction the hot water was allowed to act on the charcoal for some length of time, even as much as eight hours. It is obvious, therefore, that blood charcoal "purified by acid" is not suitable as an adsorbent of the cationic elements, sodium and potassium, from their solutions, for it does not adsorb these from their salts nor when they obtain as hydroxides. Even if the charcoal were absolutely pure, that is, free not only from adsorbed acid but also from ash residue, it would not be suitable, for Miller found that activated charcoal made from pure sugar by carbonisation adsorbed acids, but not strong bases or salts of these, and that from the latter the anionic portions were adsorbed while the cationic were retained in the solution as hydroxides.

One had, therefore, to determine whether some other material which could be purified, not necessarily absolutely, but to a degree that would prevent the results from being open to question, could be used as an adsorbent for potassium and sodium from solutions of their salts. Such an adsorbent is pure silica sand of artificial origin. This was not obtainable, except in amounts which would not suffice to use in the experiments. Attention was turned to silica sand of natural origin. A large quantity, purporting to have come from Belgium, was obtained, analyses of which, as reported, indicated: silica 99.26, Fe_2O_3 , 0.04; Al_2O_3 , 0.36; CaO 0.06; and MgO 0.08 per cent. It was in fact less pure than these analyses indicated, for in it were traces of dust derived, in previous handling and storage, from the air. A portion of the material constituting this dust was, of course, carbon, but this included inorganic salts which, added to those present in the sand originally, rendered it more impure.

A quantity of this sand, amounting to about 1900 grams, was, in successive portions, treated with hot concentrated hydrochloric acid for 30 minutes, the acid removed and the sand repeatedly washed with distilled water until the washings gave not the slightest reaction for chlorine with silver nitrate solution.

The sand, thus treated, after being dried was white with a very faint grey tinge. Under the microscope the particles were transparent, but occasionally a particle had a superficial brown tinge, such as to suggest that on its surface was a layer of silicate of iron.

To determine how far the treatment with acid had purified the sand, 60.016 gm. were heated in a large silica capsule for 20 minutes with concentrated nitrohydrochloric acid, the acid then decanted, the sand repeatedly washed with distilled water, the washings added to the acid, and the whole evaporated to dryness in another silica dish. The solids so obtained amounted to 0.031 gm. (= 0.0516 per cent.) and consisted of ferric salts (0.0245 gm.), chlorides of calcium, sodium and potassium (0.0065 gm.). In these latter the chlorides of sodium and potassium were almost negligible in amount, representing 0.002 and 0.006 per cent. respectively of the amount of sand used for this analysis.

The fact that 99.948 per cent. of this sand remains unaffected by hot nitrohydrochloric acid, and the further fact that the amounts of potassium and sodium salts present in it are so minute as to be practically negligible, indicated that this sand, after treatment with hot concentrated hydrochloric acid as described, would serve as an adsorbent, as effectively as absolutely pure silica sand, to determine the adsorption relatively of the sodium and potassium from solutions of their salts. The salts of iron and calcium which may be present in the solutions of the chlorides of potassium and sodium after they have been used with sand cannot obtain in such concentrations and amounts as found in extracts of the sand made with hot nitrohydrochloric acid, which, as a solvent, is intensely more active than solutions of the chlorides of potassium and sodium; but in any case these salts can be eliminated before determinations of the amounts of the sodium and potassium are made in the solutions after adsorption takes place. There is no aluminium salt present, there are traces of calcium and the most abundant impurity is formed of iron salts, with traces of carbon which are, in all probability, derived from the dust of the air, which cannot be excluded when the purified sand is dried in large quantities (*e.g.*, 1.5 kilos.).

With such impurities there must be a reaction between them and the chlorine ions of the solution, involving some exchange between them and the anions of the impurities. This would not involve the cations, potassium and sodium, which remain in solution and are accordingly available for adsorption.

There is also one special property of silica sand which makes it of service in the determination of the effect of ionic mobility on adsorption. This is, when

it is in contact with water, its electro-negative charge. This would attract to interfacial surfaces formed by it with water or solutions, only electro-positive ions, and, therefore, effectively those of potassium and sodium.

The sand, treated as indicated with hot concentrated hydrochloric acid, thoroughly freed from soluble salts by washing with distilled water, and then dried, was used in all the experiments of this investigation in a manner now to be described.

The sand, about 1480 gm. in amount, was placed in a Pyrex glass cylinder tube, closed at one end, the base, and open at the other, of 37 mm. inside diameter, and 97 cm. height. At the base was an inlet tube of 5 mm. inside diameter. The sand filled this cylinder to a height of 88 cm. Attached to the inlet tube at the base, and provided with a clip, was a rubber tube connected with a bulb funnel of 1.3 litres capacity, held at a level about 4 m. above that of the inlet tube. This bulb funnel was filled with the solution of the chlorides of sodium and potassium, the adsorption of which was to be determined. When the compression of the clip on the rubber tube was decreased, the solution flowed through the inlet tube to the sand, and from the base of the column of the same to the top, at a speed controlled by the amount of compression exercised by the clip. If there was no compression at all the fluid quickly reached the top of the column of sand. By graduating the compression sufficiently, the time taken for the solution to reach the top of the column of sand was what one willed, ranging up to 50 or 60 minutes. In a number of cases the compressing clip was relaxed every 5 minutes or so to allow the fluid to rise at once in the sand, about 8 cm. or so each time. In all cases the passage of the fluid through the uppermost 8-10 cm. of sand to the free surface was delayed about 5 minutes, in order to give this part of the column time to exercise to the fullest extent its adsorbent powers. Then the compression was lessened to allow the fluid to flow out on the free surface of the sand, and when as much as 40-81 c.c. had there accumulated it was transferred with a pipette to a platinum dish, in which it was carefully weighed, and this quantity analysed to determine the concentration of the sodium and potassium in it. Of some of the quantities removed thus a portion in a number of cases (about 10 c.c.) was used to determine its pH, and the remainder weighed and subsequently analysed.

The solution used in the majority of the experiments made was one of the chlorides of sodium and potassium in which the concentration of each electrolyte was 0.05 N. A solution of these two electrolytes containing 0.7614 per cent. of the chloride of sodium and 0.0381 per cent. of the chloride of potassium

was also used. After each experiment the sand was percolated with distilled water from the bulb funnel at a rate which, after 8–10 litres had been so used, removed completely the chlorides from the sand. This removal was indicated when a quantity of the water issuing from the sand and the open upper end of the containing cylinder gave not the slightest reaction for chlorides on the addition of about 5 c.c. of N AgNO_3 solution. The sand thus washed was put in a large porcelain evaporating dish, which was then placed in an electric oven and kept there at a temperature of 110°C . for 48 hours. The dish and the contained sand were then removed from the oven and when the temperature of the sand had fallen to that of the room it was kept free from contamination with dust as far as possible, and used in the next experiment.

Careful analysis of the quantity of the percolate obtained in each experiment after its weight had been accurately determined was made in the manner now to be indicated. The percolate itself was not in any case an absolutely pure solution of the chlorides of sodium and potassium. Salts of aluminium were absent, or at most in such amounts as to make them indeterminable. Calcium salts in traces were present, as was ferric chloride, but in no case in concentrations in which the iron (Fe) exceeded 0.0008 per cent. The calcium and the iron present may have been derived from the surface of sand particles, where, as already indicated, the presence of an iron compound (a silicate?) was predicated from the brown tint of the surface of some (very few) of these particles. They may have been derived from the dust from the air during the drying of the sand, a contamination that ordinarily would be negligible were the amount of sand used less than 100 gm., but with 1480 gm. the proportion of iron and calcium as chloride might add to, very slightly of course, the total weight of the chlorides of the percolate when the latter is evaporated to dryness and the solids weighed.

To remove traces of calcium and iron in the quantity of the percolate in each case to be analysed, a few small crystals of oxalic acid and about 5 c.c. of concentrated ammonia water were added to it and the solution evaporated to dryness on a hot water-bath. The solids resulting were then redissolved and the solution filtered into a platinum dish, the filtrate evaporated to dryness, the salts left heated carefully in the Bunsen flame to incinerate any oxalic acid present and to convert any trace of ferric salt into the insoluble oxide. Solution and filtration were again resorted to, the filtrate received in a weighed platinum capsule, and after 2 c.c. of concentrated hydrochloric acid were added it was evaporated to dryness on a hot water-bath. The capsule and its contents, after being kept in an electric oven at 120°C . for 4–5 hours, were

then cooled in a desiccator and weighed to determine the total amount of the chlorides of sodium and potassium present, the proportions of each of which were next ascertained.

The determinations of the amount of sodium chloride present cannot be directly made. The method for the estimation of sodium, involving the use of potassium pyroantimoniate, introduced in recent years by over-enthusiastic but by no means too-critical workers, gives (as the author has found on a thorough trial of it) uncertain, at times very erroneous, results. Besides, it introduces into the mixture of the two salts an additional quantity of potassium, which prevents the direct estimation of the original amount of potassium therein. The only possible way of determining accurately the amount of sodium chloride in the two salts is, after the amount of potassium chloride is directly found, by subtracting this amount from the total of the two salts.

The determination of the amount of potassium chloride was made with a method which the author has used in exceedingly numerous determinations of the potassium in the blood plasma of vertebrates and invertebrates, in ascitic, pleuritic, hydrocele and cerebrospinal fluids, and in the cells and tissues of vertebrates, all made in the last 26 years. This method, which has given results proving its value, has been described (1910), but how it was used in this investigation may be briefly outlined here.

The solution of the two salts was transferred to a porcelain evaporating dish, enough of a 10 per cent. solution of platinum chloride, to furnish more of this chloride than sufficed to combine with all the potassium chloride present, was added and also about 5 c.c. of concentrated hydrochloric acid. This fluid was now evaporated on a water-bath to dryness, care being taken to avoid absolute desiccation. To this 30 c.c. of absolute alcohol were added, the mixture stirred with a glass rod, then allowed to stand for an hour, after which 15 c.c. of ether were added and this alcohol and ether allowed to extract for 1 hour more. The two were then removed by decantation, a fresh quantity of alcohol and ether made in the same proportions was added, allowed to extract for an hour, then removed by decantation, and again a like quantity of alcohol and ether was allowed to extract for another hour. A third decantation followed and the dish with the residue was now placed in a warm oven with a temperature of 60° for 2-3 hours, or under a bell-jar at ordinary temperatures for 24 hours, to remove the last traces of alcohol and ether in the residue.

In the dish, and covering its contents, was now placed an inverted glass funnel, into this funnel was passed a stream of dry hydrogen gas, which, when the dish and contents were kept for 20 minutes at 250° C., reduced the platinum

of the double salt, K_2PtCl_6 , to the metallic condition. To make certain that all the platinum salt was so reduced, the dish was allowed to cool, about 5 c.c. of water was added to the residue to dissolve and set free from the reduced platinum any of the platinum salt which had escaped reduction. After evaporation the residue was again subjected to reduction for 15 minutes in a stream of dry hydrogen gas, at $250^\circ C$.

After the addition of hot water to the residue, the reduced platinum was filtered off with an ashless filter paper, which was dried, incinerated, and the platinum carefully weighed. As the potassium platinum chloride before reduction obtained as K_2PtCl_6 0.76374, multiplied by the weight of the reduced platinum, must represent the amount of potassium chloride in the original mixture of sodium chloride and potassium chloride; the amount of sodium chloride was, then, the difference between the total weight of the two chlorides and that of the potassium chloride alone as found.

How satisfactorily this method of determining the concentrations of sodium and potassium in solutions of their chlorides serves may be inferred from the results obtained with it in quantities of a solution of these chlorides in which the concentration of each was 0.05 N. The results of six such determinations, represented in the proportions NaCl : KCl, were as follows:—

	NaCl	:	KCl.
1	100		127.2
2	100		127.13
3	100		127.7
4	100		127.3
5	100		127.69
6	100		127.9

The mean of all these is NaCl : KCl :: 100 : 127.48. The actual proportions were 100 : 127.541. The experimental errors are, it must be conceded, of a very small order of magnitude.

With such a method the determinations of the potassium chloride and sodium chloride in 10 percolates were made, and the results are indicated in Table I. These are not given in the order in which each percolate was obtained, for No. 2 was the first, No. 8 the second, No. 10 the sixth, and No. 1 the last of this order. They are given in the order of the increase in the adsorption of the potassium, as well as of the length of time, ranging from 30 to 60 minutes, during which the solution furnishing a percolate was allowed to pass from the base to the summit of the column of sand.

Table I.—Adsorption with 0.05 N NaCl + 0.05 N KCl.

No.	Quantity of percolate used for analysis.	NaCl : KCl.	Percentage of NaCl in percolate.	Percentage of KCl in percolate.	pH.
1	47.390	100 : 119.96	0.2935	0.3521	3.8
2	81.019	100 : 113.72	0.3045	0.3463	3.8
3	34.149	100 : 113.21	0.3042	0.3444	—
4	46.782	100 : 110.75	0.3281	0.3634	3.5
5	73.440	100 : 110.18	0.3016	0.3323	—
6	59.638	100 : 108.89	0.2957	0.3220	—
7	36.595	100 : 107.28	0.3034	0.3255	—
8	37.254	100 : 106.45	0.3166	0.3370	—
9	40.750	100 : 105.87	0.3131	0.3315	3.2
10	57.336	100 : 104.70	0.3101	0.3247	—

From an examination of the results given in Table I it may be seen that in every instance there apparently occurs adsorption of potassium only. This is shown in the amount of the chloride of the same, in every case less than 0.05 N, in each determination. That the chlorine ions associated with potassium ions adsorbed were not so involved was indicated by the distinctly acid reaction of a portion of each percolate tested for its pH. This latter was in a number ascertained colorimetrically, but as such determinations in each case involved a considerable possible range of error, only those made potentiometrically are given. As the pH of the solution before percolation was 5.5–5.6, the change in the percolate to 3.8, 3.5 and 3.2 indicates the formation of an acid, hydrochloric, derived from the chlorine ions set free when potassium ions were adsorbed on the electro-negative surfaces of the sand particles.

That sodium ions were not adsorbed cannot be positively maintained, but it is highly probable. The reason for this lack of certainty is that in every case there occurred adsorption of water, as shown by the increase in concentration of the sodium chloride, which before percolation was 0.29227 per cent. (0.05 N), but in all the percolates it exceeded this, and by as much as 12.2 per cent. in No. 4. The diminution in volume of the solvent, thus brought about, in each percolate would mask any evidence of the adsorption of sodium ions if such adsorption occurred, but in any case it must have been absolutely negligible when contrasted with the adsorption of the potassium ions which, as indicated by the alteration of the ratio Na : K ranged from the minimum of 5.94 (No. 1) to 17.9 (No. 10) per cent. of the concentration of the potassium in the solution before percolation.

The potassium adsorbed must have been associated with HO ions set free from dissociated water molecules, the H ions from which, combining with the

Cl ions, would form the hydrochloric acid of the percolate. This would account for a minute part of the concentration of the latter. The exceptionally high concentration of No. 4 was undoubtedly due to the fact that sand, after being heated and then cooled, was at once used in obtaining this percolate, whereas two weeks or more elapsed between the heating and the cooling of the sand and its use in making the other percolates. Apparently water vapour had time to condense on the sand particles and thus decrease the amount of water adsorbed in these percolates.

If the percolate had passed up through a column of sand of very much greater length than that used in these experiments, it is extremely probable that the adsorption of potassium would go so far as to approach the elimination of it from the percolate. Before, however, that result could obtain, one would expect to find that the sodium would begin to be adsorbed and to take the place of some of the potassium in the adsorpt in the uppermost levels of the column, for, however great the adsorbability of the potassium ions, when they are greatly fewer than those of sodium, the latter would crowd to the interfaces to mingle there with those of potassium. In such adsorption layers one would expect that the percentage of the total potassium adsorbed would exceed the percentage of the total sodium adsorbed.

This is borne out in the results of experiments on the adsorption of sodium and potassium from a solution of sodium chloride 0.7614 per cent. and potassium chloride 0.0381 per cent. This solution was made to represent the average concentration of the sodium and potassium in human blood plasma, as determined by analyses made by the author. The sodium in such a solution should be 0.300 per cent. and the potassium 0.020 per cent. Two analyses of the sodium and potassium in this solution, made after the manner described above, gave (a) Na, 0.3004; K, 0.01998; and (b) Na, 0.2995; K, 0.0202 per cent. The sodium was fifteen times as concentrated as the potassium and the possible sodium ions were at least as many as twenty-six times the number of the possible potassium ions. In determining the results of adsorption from such solution, the method of percolation and the column of sand were the same as when 0.05 NaCl + 0.05 KCl was used. The results are given in Table II.

Here it may be seen that both sodium ions and potassium ions were adsorbed on the surfaces of the sand particles, but the percentage of each adsorbed was not the same, for that of potassium exceeded, and except in No. 1, was more than double that of the sodium.

It, of course, must be understood that these percentages of adsorption are not absolute. Just as in the experiments with 0.05 N NaCl + 0.05 N KCl

Table II.—Adsorption with a Solution containing 0.7614 per cent. NaCl and 0.0381 per cent. KCl.

No.	Quantity of percolate used for analysis.	Per cent. NaCl.	Per cent. KCl.	NaCl adsorbed per cent.	KCl adsorbed per cent.
1	52.217	0.72395	0.03539	4.92	7.1
2	65.136	0.7342	0.03459	3.58	9.21
3	41.88	0.74029	0.03519	2.77	7.64

there must have been adsorption of water, but how much cannot be even approximately ascertained, for the sodium and potassium ions of both electrolytes were adsorbed, whereas in percolates obtained with 0.05 N NaCl + 0.05 N KCl only the potassium ions, apparently, were adsorbed, leaving the increased concentration of the sodium chloride in the percolate to indicate the adsorption of water, though, as already postulated, not necessarily quantitatively. The percentage of adsorption given in Table II must, therefore, be regarded as relative.

A like adsorbability of potassium was shown when a solution of lithium chloride and potassium chloride was made to percolate through the column of sand. A solution, designed to be 0.05 N LiCl + 0.05 N KCl, was made and the concentration of the lithium and potassium in it was determined by the same method as used for determining the concentration of sodium and potassium in the 0.05 N NaCl + 0.05 N KCl solution. The availability of this method is rendered possible by the fact that Li_2PtCl_6 is as soluble in alcohol and ether as is Na_2PtCl_6 . Two analyses of this solution gave 0.3732 and 0.3726 (= 0.05 N) per cent. KCl and, accordingly, 0.1922 and 0.1928 (= 0.04547 N) per cent. of LiCl.* In this solution the average ratio of the LiCl to the KCl was 100 : 193.68. Percolation of the column of sand was made three times and the analyses of the percolates gave the results shown in Table III.

* The hygroscopic character of lithium chloride and its retention of water, even when kept for 24 hours at 120° C., make it difficult to prepare solutions of it having a 0.05 N concentration, and consequently also great care has to be exercised in determining the amount of the two chlorides in the solutions and the percolates containing them.

Table III.

No.	Quantity of percolate used for analysis.	LiCl : KCl.	Percentage of LiCl in percolate.	Percentage of KCl in percolate.
1	41.671	100 : 177.76	0.2000	0.3555
2	42.521	100 : 174.06	0.2001	0.3483
3	36.841	100 : 160.88	0.2081	0.3348

Here also we see evidence of the adsorption of the solvent, for the change in concentration of the LiCl in No. 3 from 0.1928 to 0.2081 per cent. involves an increase of 7.35 per cent. If the potassium were not adsorbed the chloride of it would be equally concentrated, that is, 0.40306 per cent. : but since the concentration determined was 0.3348 per cent., the potassium adsorbed must have been at least 16.9 per cent. of what was originally present. This is very close to the amount of potassium adsorbed (17.9 per cent.) from 0.05 N NaCl + 0.05 N KCl (No. 10, Table I). It is probable that a greater adsorption would have been found had more than these percolations been made.

From all these results it is certain that potassium, when in solution with either sodium or lithium and in concentration equal to or greater than that of either of the two latter, excludes them from interfaces and is adsorbed there alone, or if either sodium or lithium is also adsorbed it must be only in absolutely negligible amounts. What other factor than ionic mobility can be involved in this predominance in adsorption of potassium over sodium or lithium?

This predominance in adsorption of potassium over sodium is of fundamental importance in the relations of these two elements to living matter. Where in any system the potassium present equals or exceeds in concentration that of the sodium also present, then potassium alone is adsorbed at the interfaces, or if sodium is adsorbed it can only be to an utterly negligible extent. As the living cell, animal or vegetable, is such a system with interfaces in and on it, and as in it the potassium always exceeds in concentration the sodium, then potassium only is adsorbed at such interfaces and sodium is absent from them. The significance of this absence of sodium from the interfaces will be discussed below.

There remains the fact that in many animal and vegetable cells, examples of which have been already cited, all the potassium present is only found at interfaces. How can this be explained, if at all?

In the experiments on adsorption by sand with 0.05 N NaCl + 0.05 N KCl

the maximum amount of potassium adsorbed was only about 17·9 per cent. of the amount present in the same quantity of the solution before percolation. It was, however, stated above, and this inevitably follows from the facts advanced, that if the column of sand through which the percolation occurs were greatly increased in length the amount of potassium adsorbed from the percolating solution would tend to approach the total amount in the same quantity of the solution before percolation. Even then, with a much more greatly increased length of sand column, the potassium would be adsorbed much more, proportionally, than the sodium. This is conclusively shown in the results given in Table II. obtained with a solution of the chlorides in which the sodium ions, if complete dissociation obtained, must have been approximately twenty-six times as abundant as the potassium ions.

It is, however, not possible to estimate approximately the extent of the interfacial area in and on a living cell, and thus to be able to say that in a cell in which all the potassium present obtains at its interfaces only it so occurs because of the extent of the interfacial area involved. It can, however, be maintained that in a cell of the liver, kidney or pancreas, which is very, nay exceedingly, minute as compared with the ordinary sand particle typical of the particles of the sand used in the experiments detailed above, the extent of the interfacial area must be relatively much greater than that on a sand particle, and thus, in a small mass of cells, the quantity of potassium adsorbed would approach and even exceed, perhaps greatly, in amount that adsorbed by a very much greater volume of sand.

This would account for a considerable amount of the concentration of the potassium at the interfaces in cells. The remainder of the concentration, and the absence of potassium from the general cytoplasm of the cells in the instances cited, can only be due to the character of the constituents of the interfaces.

There is the generalisation, derived from the results of a wide range of experimental work, that chemical activity is greater, often much greater, at interfaces than in the general volume of the solution systems in which they obtain. This, it may be maintained, is true in a system like the living cell. In it the greatest chemical activity must occur at the interfaces, involving chemical transformations resulting in the formation, among other products, of ionic elements of an organic type having the property of lowering the interfacial or surface tension, and capable of combining with the potassium present and that diffusing to the interfaces from the cytoplasm elsewhere in the cell. In this diffusion, because of higher ionic mobility, the potassium ions would outstrip those of sodium and accumulate at the interfaces, tending to

decrease the interfacial hydrogen ion concentration. If anionic metabolites, also having the property of lowering the surface tension, are produced in the general body of the cytoplasm, these, by uniting more readily with the potassium, because of its greater ionic mobility, than with the sodium in the cytoplasm, would sweep all the potassium to interfaces, with the result of tending to concentrate all the potassium of the cell at such interfaces.

One may suggest also that in this concentration of the potassium at the interfaces in the cell its properties as an element, as compared with those of sodium, play a part. The two elements are of course monovalent, and one can replace the other in ordinary chemical reactions, but potassium is much more active than sodium in the production of a number of organic compounds, can unite with or promote the formation of compounds unlike those formed with sodium under the same conditions, and can even form compounds in the production of which sodium is inert.

Some examples to illustrate this difference in reactivity may be cited. Ethyl aldehyde is condensed by potassium salts to aldol, by sodium salts to crotonic aldehyde. When metallic potassium is dissolved in pyrrol potassium pyrrol (C_4H_4NK) is formed, which also is formed when boiling pyrrol is treated with solid potash (Anderson), but metallic sodium forms with pyrrol sodium pyrrol only at very high temperatures, while soda does not at all react with pyrrol (Ciamician and Dennstedt). Potassium will react with benzene at high temperatures, forming potassium benzene (Abeljan) but sodium does not combine with benzene at any temperature.

There is also the radioactivity of potassium, due to the isotope which constitutes slightly more than 5 per cent. of it, a property which Zwaardemaker has stressed in explaining the special part that potassium plays in living matter. Whether, however, it accounts for the reactive power of potassium, as compared with that of sodium, and whether it promotes in any degree its adsorption in living matter, cannot be determined, as there are no facts known bearing thereon.

There can, however, be no doubt that the ionic mobility of potassium accounts in very great part for the concentration of this element at interfaces in the many instances of cells, animal and vegetable, in which the author has investigated the distribution of potassium. Furthermore, at these interfaces where chemical activity must be more pronounced than elsewhere in such cells, and where sodium, if it obtains at all, can be present only in negligible traces, this activity must be controlled, in part at least, by the potassium present.

It is possible also that at portions of the surfaces of the cells where inter-

facial activity is great the ionic mobility of potassium may cause it to diffuse into the cells more readily than sodium from media (plasma, lymph and sea-water) in which the concentration of sodium greatly exceeds that of potassium. This greater diffusibility of potassium must be considered in any attempt to account for the concentration of this element, as compared with that of sodium, in all cells, animal and vegetable.

Summary.

1. From a solution of potassic chloride and sodium chloride of equimolecular concentration, when silica sand of natural origin was used as an adsorbent, potassium alone was adsorbed. With a solution of lithium chloride and potassium chloride of approximately equimolecular concentration the same result was obtained.

2. The amount of potassium so adsorbed depends on the length of time allowed for adsorption to take place and on the quantity of the sand through which the solution of the two salts is percolated. With a 0.05 N NaCl + 0.05 N KCl the potassium adsorbed thus ranged from 5.68 to 17.9 per cent. of that in the solution before percolation.

3. With a solution in which the sodium chloride was 0.7614 per cent. and the potassium chloride 0.0381 per cent. (the concentrations of these which obtain in human blood plasma) both sodium and potassium were adsorbed, but the percentage of the total potassium present so adsorbed by the silica sand exceeded, and in several instances by more than twice, the percentage of the total sodium present so adsorbed, although the possible sodium ions present in the solution were twenty-six times the number of the possible potassium ions therein.

4. These results are explained as due to the high ionic mobility of potassium as compared with the ionic mobility of either sodium or lithium. That of potassium is 65.3, that of sodium is 44.3, and that of lithium 35.5 at 18° C. In solutions of potassium chloride and either sodium chloride or lithium chloride, of equimolecular concentrations, the velocity of migration of the potassium ions causes them to reach first and then occupy, thus to the exclusion of the sodium ions or lithium ions, the interfacial surfaces. This would, consequently, happen also when the molecular concentration of the potassium exceeded that of the sodium in the solution. When the concentration of the sodium greatly exceeds the concentration of potassium in a solution, the velocity of the migration of the ions of the latter explains why more of them are adsorbed, relatively, than are those of sodium.

5. It would appear that chlorine is not adsorbed on silica sand, for in certain of the percolates made from 0.05 N NaCl + 0.05 N KCl the pH ranged from 3.8 to 3.2, while the pH of this solution before percolation was 5.5-5.6. This indicates the presence of hydrochloric acid in the percolate formed from the chlorine ions set free from the potassium ions adsorbed.

The bearing of these results in explaining many of the findings regarding the interfacial localisation of potassium in living matter is discussed in the text of this contribution.

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Sex-Reversal in a Pigeon (Columba livia).

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[PLATES 23–27.]

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1.—*Introduction.*

Although a very large number of cases of sex-reversal in birds have been described during the last few years, very little is known as to the exact origin and development of the spermatic tissue. This deficiency is due to the fact that it is rare to find the early stages of the process in a gonad which also shows more definite and advanced stages. The present instance is of unique interest on account of containing all stages in the process of development of spermatic tubules in the ovary. It is also of special interest in showing the various prophase stages of the maturation division in the tubules and thus placing their spermatic nature beyond dispute. Previously spermatogenesis has only been observed in a very few sex-reversed birds (Crew (2), Zawadowsky and Zubina (9), etc.). Natural, as distinct from experimental, sex-reversal in birds almost invariably accompanies a pathological condition of the ovaries, such as ovarian neoplasm or tubercle. No disease, however, is apparent in the present instance, which appeared healthy in every way. Furthermore there appears to be only one previous case of sex-reversal in a pigeon on record (Riddle (7)), and in that instance the gonads were not available for description.

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II.—*General Description.*

A pigeon (A 975), together with a number of others, came into the possession of the Department of Biochemistry, University College, London, on December 6, 1927. The bird appeared normal in every respect and weighed 554 grams. It was immediately included, as a control, in a feeding experiment and was forcibly fed on artificial vitamin B deficient diet, with an adequate amount of yeast extract, daily for 27 days. At the end of that time it was still apparently healthy and weighed 511 grams. It was killed on January 2, 1928, and an attempt was made to remove the suprarenal glands. It was found that a large tumour-like swelling occupied the site of the gonads and suprarenal glands, the major portion being situated on the left side and partly separated from a smaller portion on the right side by the vena cava (Plate 23, figs. 1 and 2). On dissection a well-developed oviduct was seen on the left side, but none was noticed on the right side nor were vasa deferentia observed. The "tumour" was then removed and fixed in Bouin's fluid while the rest of the body was thrown away.

The "tumour" was photographed and portions were sectioned and stained with Ehrlich's hæmatoxylin and eosin, Heidenhain's iron-hæmatoxylin or Pasini's stain. The material proved to be beautifully fixed and well suited to histological and cytological study. Histological examination showed that the "tumour" consisted of a large testis on the left and a smaller ovotestis on the right. The region between these was composed largely of connective tissue, but included a nodule of suprarenal tissue, a portion of the kidney and the wall of the vena cava. The relations of these parts can be seen by comparing Plate 23, figs. 1 and 2.

The left gonad was a smooth lobed organ measuring 27.5 mm. long. The posterior portion was broader and deeper than the anterior. The surface appeared well vascularised. This gonad was attached along its dorso-median border to the connective tissue surrounding the vena cava. One or two small rounded protuberances near its anterior extremity, shown in the photograph, were suggestive of oocytes. Sections were accordingly cut across this area in two directions, indicated by dotted lines in the last figure. In addition longitudinal median sections were made, which are not indicated, as they were in the same plane as the illustration. The gonad consisted almost entirely of testis tissue, arranged in lobules within thin connective tissue trabeculæ. The

spermatic tissue consisted of spermatic cords and tubules in varying stages of development.

A few degenerating oocytes were scattered superficially near the anterior end. The whole gonad was surrounded by a thick fibrous tunica albuginea, continuous with the connective tissue trabeculae which separated the lobules. A number of tubules, resembling vasa efferentia, were grouped in this fibrous tunica at the anterior end on the medial side.

The right gonad was a long cylindrical organ, parallel to the left gonad, with a large pendant ventral lobe attached near its anterior end. The cylindrical portion, which was partly embedded in the connective tissue surrounding the vena cava, measured 15 mm. long, and its surface was lobulated. It consisted of ovarian tissue containing many small oocytes in various stages of development, and here and there islets of large clear cells scattered in the stroma or forming small groups, and one or two lobules of spermatic tubules. These clear islet cells were destined to form the spermatic cords. The pendant lobe measured about 7 mm. long and its surface, which was smooth, resembled that of the left gonad.

The tissue composing this lobe was largely spermatic but included a few groups of degenerating oocytes. It closely resembled the left gonad, except that the spermatic cords and tubules were on the whole in an earlier stage of development. The groups of degenerating oocytes were chiefly superficial and were always surrounded by forming sex-cords and embryonic spermatic tubules.

III.—*Histological Description.*

A. *The Structure of the Ovary.*—The ovarian portions of the gonad are full of small oocytes ranging up to about 0.25 mm. in diameter (Plate 24, fig. 2). The majority of these show more or less marked signs of degeneration, but a few appear normal and to be surrounded by normal follicles. The largest of these follicles have a single layered membrana granulosa composed of slightly flattened cells, with a clear protoplasm, and full well-stained nuclei. They rest on a well-marked basement membrane or membrana propria which stains intensely blue with Pasini. The theca, outside the membrana propria, is in the form of a concentric zone of stromal cells, some of which appear to be connective tissue fibroblasts, while others, less elongated, might be considered either as connective tissue or epithelial elements. The smallest follicles have a similar theca and an equally well-marked membrana propria, but the membrana granulosa is very thin and is composed of cells which are more flattened than in the larger ones.

The vast majority of the oocytes exhibit obvious signs of degeneration. The earliest signs consist of shrinkage and consequent crumpling of the nucleus accompanied by vacuolation of the cytoplasm. When these changes have become advanced the whole oocyte collapses, shrinking away from the follicular wall around the major part of its periphery. This collapse results in a cavity being formed in the follicle, in which the irregular crescent-shaped oocyte is free or partly free. The shrinking oocyte appears, in all cases, to tear away the entire follicular epithelium from the wall of the follicle. The follicular epithelium remains attached to the degenerating remains of the oocyte and consequently itself degenerates *in toto*. A part of the theca is also torn away in many cases, and sometimes the entire theca appears to be separated in this manner from the surrounding stroma (Plate 26, fig. 1). The theca in other cases remains intact, the line of separation being between it and the follicular epithelium. Finally the oocyte, follicular epithelium and any of the theca which may have been torn away from the wall degenerate completely, leaving only a more or less collapsed cavity (Plate 25, fig. 25) in the stroma to mark the former site of the follicle. This process of follicular degeneration is slow and accompanied by remarkably little, if any, leucocytosis.

It is impossible to identify with certainty the so-called "luteal" or "interstitial" cells of the ovary, since the material was fixed in Bouin's fluid and not in an osmic fixative. Many large clear cells, however, are present in the stroma which clearly resemble "interstitial" cells. They occur as islets singly or in small groups, often in the neighbourhood of follicles (Plate 26, figs. 1 and 2). Sometimes they occur more or less imbedded in the theca, or in follicles in which the theca has collapsed with the degenerating oocyte, actually abutting on the cavity previously occupied by the follicle (Plate 26, fig. 1). They appear in these circumstances as if they might be derived from the elements of the theca, but this interpretation is not supported by intermediate stages and is incorrect. They are easily distinguishable by their size from the surrounding cells and will be referred to as the "islet cells." The outline is distinct and rounded while the cytoplasm is very faintly amphophil and is non-granular and finely vacuolated. They appear consequently bright and clear. The large nucleus is slightly excentric in position and is spherical and plump in appearance. The chromatin is in the form of small, but distinct, lumps at the nodes of a fine net, and is peripheral in position. One, sometimes two or three, fairly large acidophil plasmosomes are present, usually in a central position. These cells (Plate 26, figs. 1 and 2) resemble, therefore, very closely the so-called "interstitial" cells of the bird ovary as they appear in preparations fixed in Bouin's fluid.

The peritoneal or germinal epithelium which covers the entire surface of the ovarian portions of the gonad is cubical, rather columnar in places (Plate 27, fig. 1). It is identical with that of any adult pigeon and is quite normal. It exhibits nowhere any signs whatever of proliferation or of the formation of sex-cords, such as occurs in the embryonic gonad.

The ovarian tunica albuginea, situated immediately beneath the germinal epithelium is also normal. It is thin, rarely composed of more than two fibroblasts in thickness.

B. The Formation of the Sex-cords.—The chief interest attached to this case of sex-reversal lies in the fact that all stages in the formation of the spermatic tubules from ovarian elements are present. The earliest stages (Plate 25, figs. 1, 2 and 3) are to be found in the ovarian portions of the gonad and wherever traces of the ovarian structure remain. Well-formed sex-cords are present in these regions also. These early stages are absent from the major part of the testis, which is composed of spermatic tubules in later stages of development (Plate 24, fig. 5).

The sex-cords are derived from the large clear islet cells scattered in the ovarian stroma and in the thecæ of the follicles, and which appear to be the so-called "interstitial" cells of the ovary. These cells, as has been described, are scattered singly or in small groups. They appear to be normal constituents of the ovary, since they are distinct from all the other cell-elements, and because no stages in their formation from any other type of cell could be found.

These cells, as stated, are present throughout the ovarian stroma, scattered singly or in small groups of from two to half-a-dozen or even more. The stages between these small groups and definite sex-cords are limited to the zones where definite ovarian tissue merges into differentiated testicular tissue (Plate 25, figs. 1, 2 and 3). In this zone groups of increasing size can be found, until 6 to 10 cells can be seen in a cross-section of a single one (Plate 25, figs. 2 and 3). The groups of the latter size have lost their spherical shape and are lengthening into cylinders or cords. About this stage an occasional cell can be seen in the contraction stage of the typical spermatocyte (Plate 26, fig. 4). Intermediate stages between these and the larger spermatic tubules, in which many spermatocytes exhibiting contraction figures are present, are plentiful. The cells change little during the formation of the sex-cords. The cell body grows very slightly or not at all. The nucleus, however, does increase definitely in size but retains its plump rounded appearance. The islet cells in the purely ovarian regions do not exhibit mitosis. These cells in even the small sex-cords can be seen in mitosis.

The forming sex-cords acquire a definite thin connective-tissue coat of concentrically arranged fibroblasts (Plate 26, fig. 4). This coat is apparently derived from the surrounding stromal elements which become arranged in the concentric manner owing to the growth of the sex-cord and the consequent stretching of the surrounding tissues.

This origin of the spermatic tissue is very definite and distinct and the material appears to admit of no other interpretation whatever. The series of stages (Plate 26, fig. 4) is very complete or, rather, is quite continuous. Moreover, no other cells are present which resemble the cells composing the sex-cords or from which they could be supposed to originate. The germinal epithelium and the follicular epithelium are ruled out as possible sources, since the former exhibits no signs of proliferation and the latter degenerates completely.

C. *The Structure of the Spermatic Tissue.*—The spermatic tubules differ to some extent in the different regions of the gonad (Plate 24, fig. 5). They are smaller and apparently younger in the parts near the ovarian tissues and the sites of origin of the sex-cords. They are apparently normal in these regions and resemble the tubules of an immature testis. They have no definite lumen and are filled with Sertoli cells, spermatogonia and spermatocytes in various phases (Plate 25, fig. 4). The older and larger tubules often have a lumen, and contain many abnormal spermatocytes exhibiting multipolar spindles, chromatin extrusions in the cytoplasm, and multinuclear and giant-cell conditions, as well as pycnotic cells. No spermatozoa are present in any of the tubules, and it appears as though the spermatogenesis, which is normal up to a certain point, is unable to proceed beyond it. However, many apparently normal cells are present in the older tubules. All the typical reduction prophase stages are distinguishable, as well as first and second spermatocyte spindles (Plate 26, fig. 3). It was hoped at first to be able to distinguish the sex-chromosomes and to determine whether the bird was a chromosomal male or female. Prof. Ruggles Gates kindly examined the material with this in view, but could arrive at no conclusions owing to the clumping of the chromosomes.

The abnormalities of the testis of this bird, although it was suffering from no dietary deficiency, may be said to resemble those produced in a normal male bird by malnutrition or vitamin B deficiency (Marrian and Parkes (5)). The tubules are never so large as those in active spermatogenesis in a normal breeding adult.

They are surrounded and separated by thin connective tissue trabeculae which constitute a coat around each tubule (Plate 25, fig. 4). These trabeculae

form little islands of connective tissue in the interstices between the tubules. Islet cells are absent from these regions.

The testis portions of the gonad are covered by a well-developed tunica albuginea (Plate 27, fig. 2), which forms a very thick fibrous layer in places. No germinal or peritoneal epithelium is visible outside this coat. Wherever ovarian and testicular tissues meet superficially the cubical germinal epithelium of the ovary can be observed to thin out and disappear over the margin of the testis portion, at the same time as the underlying ovarian tunica thickens to pass over into that of the testis (Plate 24, fig. 3).

D. The Efferent Ducts.—A number of tubules or ducts, closely resembling the vasa efferentia of the normal epididymis are present in the thick fibrous tunica which covers the median surface of the left gonad at the anterior end (Plate 24, fig. 1). These tubules are lined by a simple cubical epithelium and have a clear lumen, sometimes containing a few pycnotic cells and a slight coagulum. They approximate very closely to the outer borders of the spermatatic tubules in places, but do not appear to communicate actually with them. An occasional strand of cells, imbedded in the connective tissue, recalls an incipient rete tubule in which the lumen is not open. It therefore appears as though the spermatatic tubules had no outlet, although apparent efferent ducts are developing.

IV.—*Discussion.*

A. The Reversal of Sex.—The evidence that the bird described in this paper was in process of sex-reversal is furnished entirely by the structure of the gonads. No other record of the bird is available. This evidence may be summarised under six heads :—

- (1) The gonads are irregular in shape, which is always the case in sex-reversals, since they have been derived from ovaries.
- (2) The left gonad is much larger than the right, as in normal females.
- (3) The ovarian tissue occurs in several places over the surface of the gonads. It exhibits everywhere unmistakable signs of degeneration.
- (4) The testes do not exhibit a uniform development of spermatatic tubules ; these are represented by sex-cords and tubules of embryonic type in some regions and by older tubules of varying age in other regions. All the tubules in a normal testis are in the same stage of development.
- (5) The sex-cords and young tubules are actively growing, while the older ones show some signs of degeneration.
- (6) A well-developed oviduct, similar to that of a normal non-breeding adult female, was present on the left side.

These considerations show that the gonads were originally ovaries and that they are in process of transformation into testes. It is interesting to note that the right gonad, though the smaller, contains a considerable amount of ovarian tissue. This is unusual in the fowl, in which the right gonad is rudimentary in the adult female and in sex-reversals generally develops into a testis, not an ovotestis. The right gonad in normal adult female pigeons is often developed and functional, according to Riddle (8). This fact accounts for the condition in the present instance.

The cause of this reversal cannot be determined, but it does not appear to be the result of the destruction of the ovary by disease, as is common in cases of sex-reversal in adult birds.

This bird is of interest in exhibiting active spermatogenesis in the spermatic tubules, which is unusual in cases of sex-reversal although it has been recorded previously in a few instances (Fell (3), Zawadowsky and Zubina (9)). The spermatogenesis appears to proceed in a normal manner up to a certain point but then becomes abnormal and fails to produce any spermatozoa. These latter abnormalities may be accounted for by the absence of outlet from the tubules, which would result in a condition similar to that produced in a normal testis by section or ligation of the vas deferens.

B. *Redifferentiation*.—The islets of clear cells in the thecæ of the follicles and in the stroma of the ovary of the bird have been described by various authors as "interstitial" or "luteal" cells. Fell (4) has shown that they are derived in the fowl embryo from the medullary cords. Their formation begins about the eleventh day of incubation. They increase in number during adult life. Nonidez (6) and Fell are agreed that this increase is not due to mitosis, but to the transformation of the epithelial elements in the thecæ and stroma, which are derived from the distal extremities of the medullary cords. Fell also described their formation from atretic follicles and, in cases of sex-reversal, from degenerating sex-cords. They are formed by a process of fatty infiltration, which resembles at first that described in the formation of adipose cells. The mitochondria multiply and transform into lipid vacuoles. These lipid vacuoles give rise to larger fat vacuoles. The fat vacuoles in adipose cells run together, and accumulate to such an extent that the nucleus is flattened against the side, but in the islet cells the fat vacuoles do not fuse, and the nucleus retains its central position and spherical shape. Their formation from abortive medullary cords or atretic follicles thus supports the cytological evidence that they are specialised elements. Whatever their true nature may be, their structure and position in the ovary are characteristic, and they are quite unlike any other category of ovarian element.

The islet cells described in this paper, as giving rise to the sex-cords, resemble the "luteal" or "interstitial" cells of other authors in every respect. They are large clear cells scattered singly or in small islets in the thecæ and stroma of the ovarian tissue. The cytoplasm is vacuolated, but whether or not these vacuoles contained fat is uncertain owing to the fixative employed. The nucleus is spherical and central or slightly excentric. Moreover they resemble no other elements in the ovary. The evidence, though not conclusive without osmic fixation, is therefore entirely in favour of the identity of these cells with the islet cells of the normal avian ovary.

The islet cells are much more plentiful in the ovarian regions of these gonads than they are in a normal ovary. This frequency is easily explained, if Fell's work is admitted, by the augmentation in their numbers resulting from the extensive degeneration of the ovarian tissue. None are formed, in the bird described, from a degeneration of the sex-cords. This is shown by the fact that wherever they are present in the vicinity of the spermatie tissue, the sex-cords and embryonic tubules are in an active growing condition and show no signs of degeneration. The older spermatie tubules in other regions do show signs of partial degeneration, but the islet cells are absent or extremely rare in their vicinity. The frequency of islet cells and the complete series of stages observable in regions where sex-cords are differentiating show clearly that the latter are derived from the former.

The islets cells do not appear to exhibit mitosis in the purely ovarian regions. This fact supports the observations of Fell (4) and Nonidez (6). Mitoses are, however, observable in even the youngest sex-cords, the growth of which is undoubtedly due to mitotic division of the cells. It is probable that osmic preparations would have exhibited a process of dedifferentiation of the islet cells, in the form of a decrease in the fat content, prior to the formation of the sex-cords and the onset of mitotic activity. In any case the islet cells must be considered as differentiated ovarian elements, and their transformation into spermatogonia as an amazing instance of redifferentiation.

Fell's work renders the interpretation of these results less startling from the embryological than from the cytological point of view, since she derives the islet, or "luteal," cells from the medullary cords. Though abortive in the ovary the medullary cords give rise to the definitive spermatie tubules in the testis. It is not unreasonable therefore to attribute a similar rôle to their derivatives in cases of sex-reversal.

The most thorough previous investigation of the origin of the sex-cords in sex-reversed birds was that of Fell (3). She demonstrated their origin from

cords proliferated from the peritoneal epithelium of the ovary and maintained that this was the chief source. She admitted, however, the possibility of a dual origin from elements within the ovary as well as from the peritoneal epithelium and suggested that if such was the case the remains of the medullary cords possibly played a part. Moreover she noted the presence of enormous numbers of typical islet cells in the gonads in process of sex-reversal. Caridroit (1) came to a similar conclusion as to the origin of sex-cords in ovarian grafts. He considered that they were derived either by proliferation from the germinal epithelium or from the thecæ, probably from cells of the medullary cords lodged there which had not transformed into islet cells ("cellules claires"). These workers, therefore, foreshadowed in part the conclusions arrived at in the present paper. The fact that no epithelial proliferation was present in the bird described affords no ground for criticising the description of the peritoneal origin of the sex-cords in their examples.

The origin of spermatogonia from the islet cells of the ovary is not in accord with the theory of the continuity of the germ-plasm. The view that the definitive germ-cells are derived entirely from the primordial germ-cells which migrate into the germinal ridge during embryonic life is meaningless in this respect, unless it is also assumed that they do not undergo differentiation into somatic cells. The material under discussion obviously affords no evidence as to whether the islet cells originated from germ cells contained in the medullary cords or from somatic cells. It is obvious, however, that the islet cells are in some degree differentiated, and if this be admitted it destroys the chief reason for assuming that they are derived from primordial germ-cells, and renders that assumption meaningless. They were, in fact, prior to transforming into spermatogonia differentiated ovarian elements, irrespective of their origin.

A minor point of interest is the occurrence of a germinal epithelium covering the surface of the ovarian portions of the gonads, but its absence from the testicular regions, where the tunica albuginea forms a thick fibrous coat. This observation shows that the disappearance of the germinal epithelium and the development of the thick tunica take place in response to the stimulation of the tissues in their immediate neighbourhood. This differentiation is not a hormonal effect, since it is not uniform.

V.—*Summary.*

1. The pigeon described had a large testis and a well-developed oviduct on the left and an ovotestis on the right.

2. The spermatic tissue proved to be actively growing and in various stages of development, while the ovarian tissue was degenerating, thus indicating that the gonads were in process of sex-reversal from ovaries into testes.

3. The sex-reversal was not the result of ovarian disease, of which there were no signs.

4. The spermatic tubules originated from sex-cords formed from the islet cells of the ovary, which were very numerous. These islet cells have been described also as "interstitial" or "luteal" cells. The sex-cords were not derived by proliferation from the peritoneal epithelium.

5. The spermatic tubules exhibited all the typical reduction prophase stages. Spermatozoa were not formed.

6. The ovarian portions of the gonad were covered by a typical ovarian peritoneal epithelium. This was not apparent over the spermatic portions where the tunica albuginea was very thick.

7. Tubules embedded in the tunica albuginea in one region resembled mesonephric tubules and were possibly developing efferent ducts.

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DESCRIPTION OF PLATES.

Guide Letters.—C., islet cells. CP., spermatocytes in contraction phase. DF., degenerating follicle. ET., efferent tubules. FE., follicular epithelium. GE., germinal epithelium. K., kidney. O., oocyte. OV., ovary. S., suprarenal. SC., sex-cord. ST., spermatic tubule. T., theca of follicle. TA., tunica albuginea. TL., left testis. TR., right testis. V., vena cava.

PLATE 23.

FIG. 1.—Diagrammatic key to fig. 2. The broken lines indicate the planes of sections.
FIG. 2.—Photograph of gonads and adjacent tissues from ventral aspect. The suprarenal marks the anterior pole of the mass. $\times 3.3$.

PLATE 24.

FIG. 1.—Microphotograph of testis showing efferent tubules developing in tunica albuginea. $\times 72$.
FIG. 2.—Microphotograph of typical ovarian region. Note absence of large oocytes and degenerate condition of most follicles. $\times 120$.
FIG. 3.—Microphotograph of junction of ovarian and testicular tissue to show ovarian germinal epithelium merging into tunica albuginea of testis. $\times 200$.
FIG. 4.—Microphotograph of ovotestis showing superficial position of ovarian relative to testicular tissue. $\times 75$.
FIG. 5.—Microphotograph of testis; general view of spermatic tubules. $\times 75$.

PLATE 25.

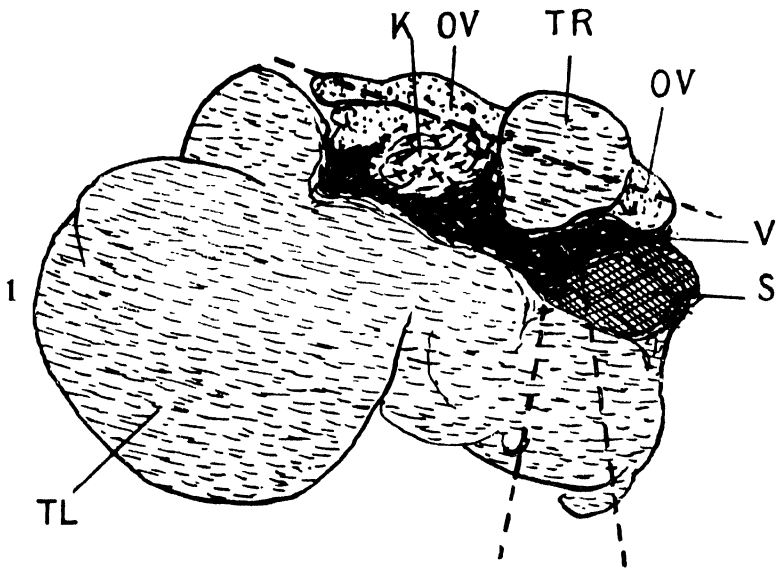
FIG. 1.—Microphotograph of ovotestis. The ovarian portion unites with the spermatic just outside the field. Showing islet cells, various stages of forming sex-cords, and spermatic tubules. $\times 200$.
FIG. 2.—Microphotograph of portion of field in fig. 1. $\times 735$.
FIG. 3.—Microphotograph of ovotestis, showing degenerating follicle, islet cells and sex-cords in various stages of formation. $\times 670$.
FIG. 4.—Microphotograph of portions of two spermatic tubules showing spermatogenesis. $\times 1000$.

PLATE 26.

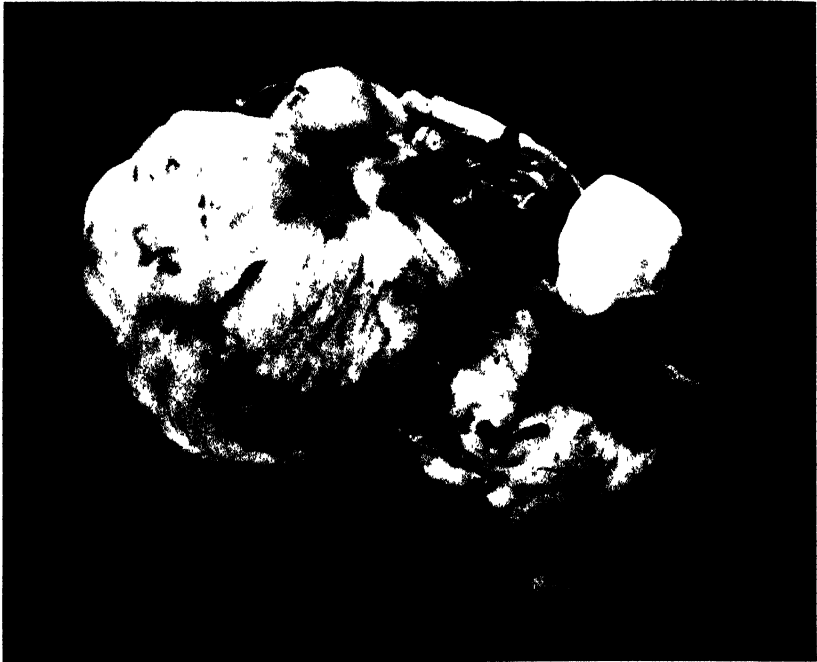
FIG. 1.—Camera-lucida drawing of ovarian portion, showing germinal epithelium, degenerating oocytes, and islet cells. $\times 800$.
FIG. 2.—Camera-lucida drawing of islet cells in theca of follicle. $\times 800$.
FIG. 3.—Camera-lucida drawing of some spermatocytes from ovotestis. $\times 750$.
FIG. 4.—Diagrammatic drawing of five stages in the formation of sex-cords from islet cells. Note prophase changes apparent in some of the cells of the last stage. $\times 800$.

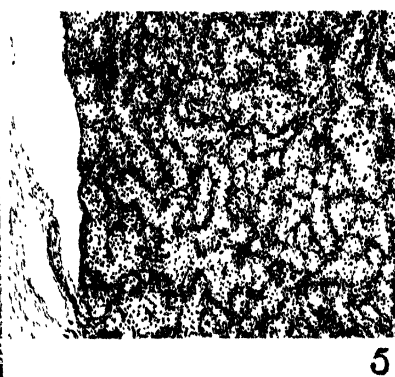
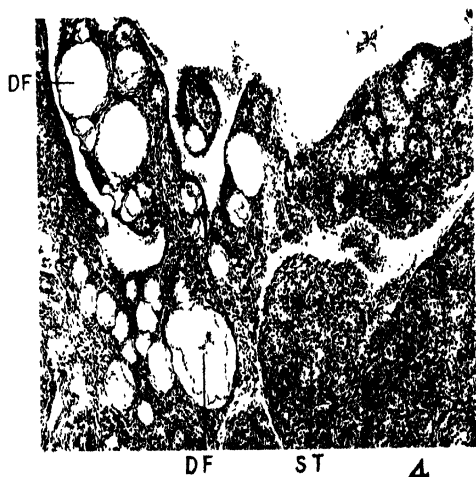
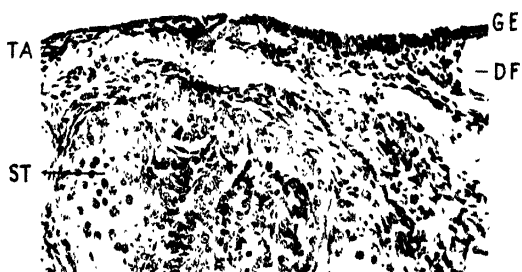
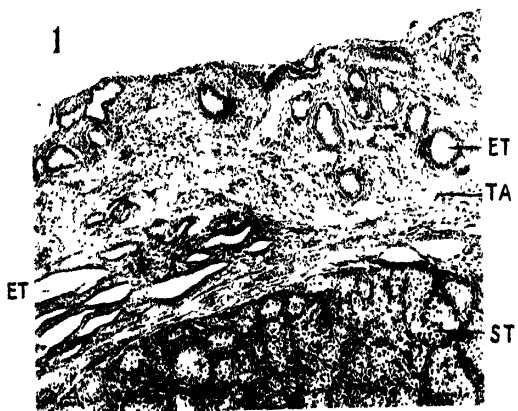
PLATE 27.

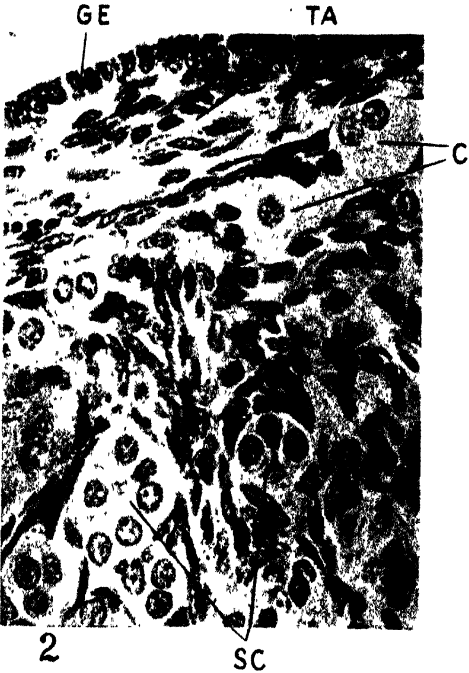
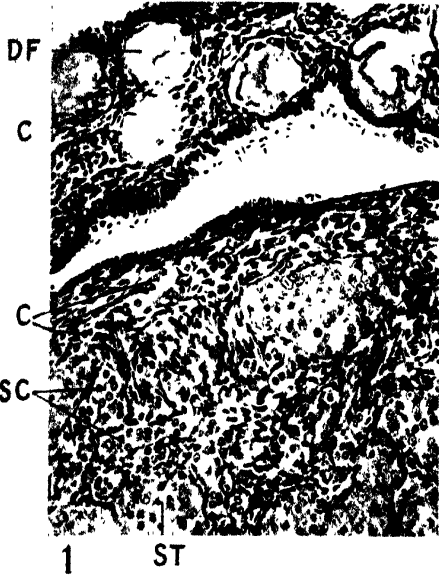
FIG. 1.—Camera-lucida drawing of ovarian region, showing degenerating follicles, islet cells and germinal epithelium. $\times 900$.
FIG. 2.—Camera-lucida drawing of testicular region, showing spermatic tubules and well-developed tunica albuginea. $\times 900$.

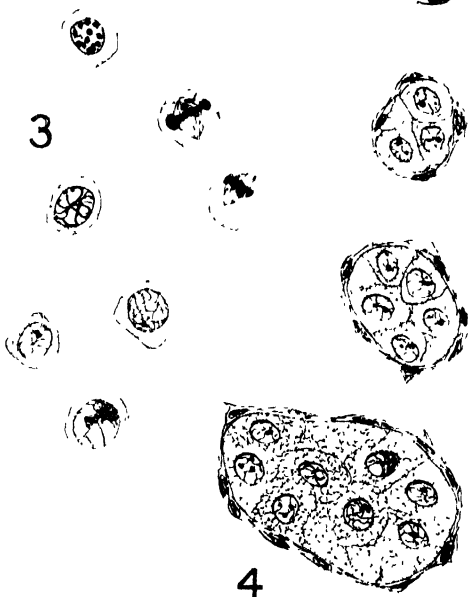
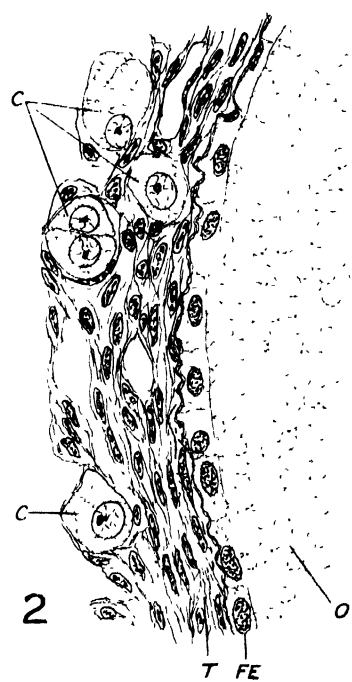
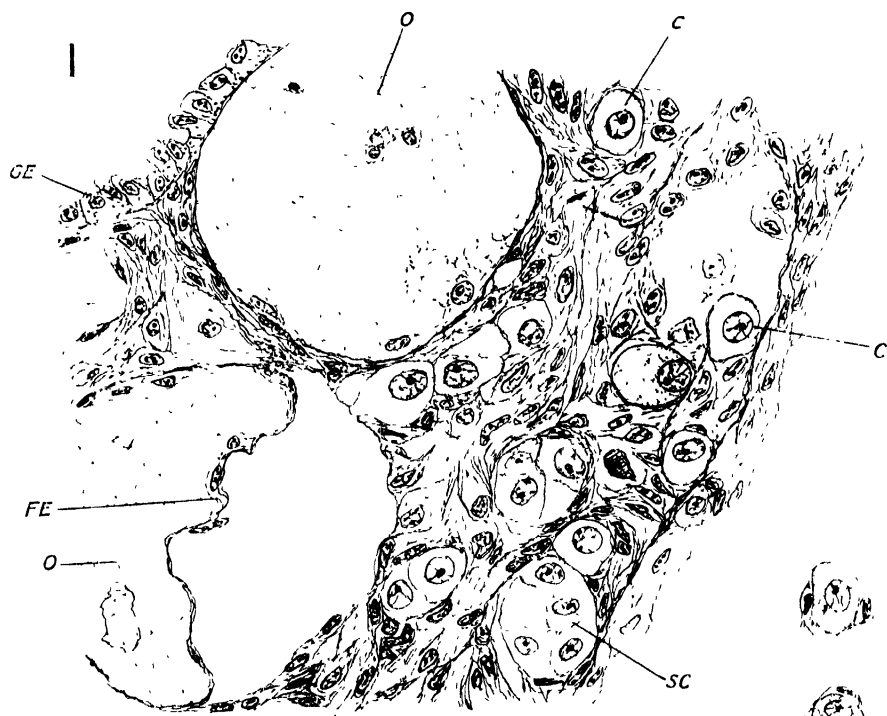


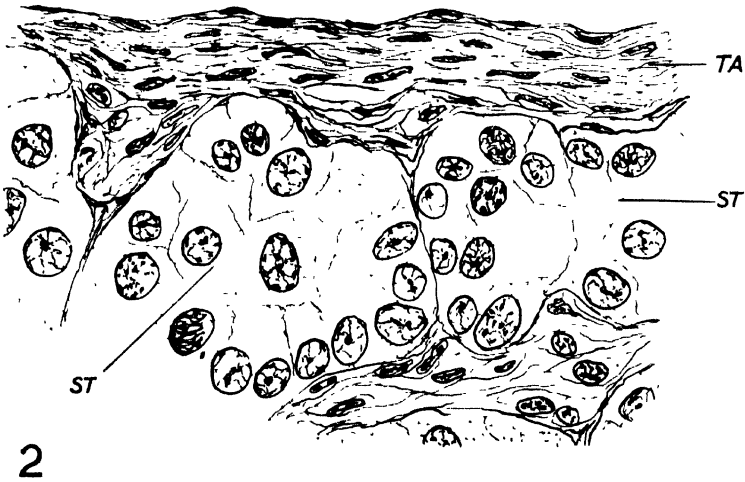
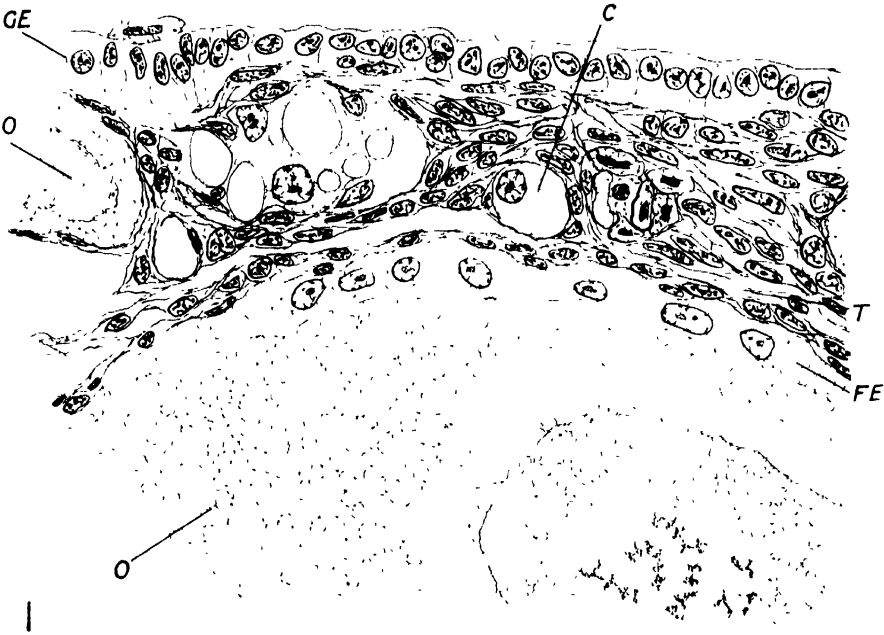
2











The Post-Nuclear Body in the Spermatogenesis of Cavia cobaya, and other Animals.

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(From the Zoology School, Trinity College, Dublin.)

[PLATES 28–30.]

Introduction.

In a number of studies on spermatogenesis of molluscs, and in the annelid *Saccocirrus*, the senior author has drawn attention to the presence of post-nuclear granules, so-called, which take part in the formation of the ripe sperm. The possibility of the presence of such bodies in all flagellate spermatozoa will seem evident from what follows in this paper. This work will serve as an introduction to a study on the effect of X-rays on the spermatogenesis of guinea-pigs and other laboratory animals, which the writers at present have in hand.

The senior author had already, in conjunction with Dr. Woodger, of the Middlesex Hospital, studied *Cavia* material prepared by Cajal's Golgi apparatus method, but the preparations which have been obtained with the chilled Da Fano method, on the lines advocated by Dr. Hope Hibbard, have been so remarkably good that it has been found possible to give a satisfactory description of these new bodies in *Cavia* spermatids.

The writers gratefully acknowledge a grant from the British Medical Association Research Funds. Some of the diagrams were made by Miss M. O'Brien, whom we thank.

Previous Work.

In 1918–19 the senior writer discovered that the spermatids of several species of pulmonate molluscs contained special bodies, which were neither mito-

chondria, Golgi bodies nor chromatoid bodies. In the case of certain forms such as *Arion*, it was possible to trace these granules back into the young spermatocyte, as shown in Plate 30, fig. 29, PNG. During the spermatocyte divisions these structures appeared to be sorted out between the resultant spermatids, because each spermatid was found to have a quarter group of these granules, situated behind the nucleus, as depicted in Plate 30, figs. 30, 31, 32. Finally the granules ran together and formed a sort of post-nuclear plate, as shown in Plate 30, fig. 33. Subsequently, in *Saccocirrus*, Plate 30, figs. 34 and 35, PNB, what are undoubtedly similar structures were described in the year 1922.

In the spermatogenesis of moths, neither Meves, the senior writer nor Vishwa Nath found such bodies, though Bowen has figured an enigmatic granule in moth spermatids, which may be the post-nuclear body. A consideration of Bowen's papers will be found in the discussion.

What seem to be the same post-nuclear structures may have been seen by Duesberg, Meves and Retzius, in their studies on mammalian sperms, but no account whatever has been given of their metamorphosis from granules into the more strongly staining and therefore more easily demonstrable bands in the adult spermatozoa.

In the various text-books on Cytology there is no recognition of a universal post-nuclear apparatus in flagellate spermatozoa, but it has not been possible for the writers to search the literature on spermatogenesis for the scattered references to such structures, as well as it is desirable. This present paper will serve its purpose admirably if it draws the attention of other workers on spermatogenesis to the necessity to look out for similar structures in the forms which they may be examining. The presence of quite definite post-nuclear granules in such widely separated organisms as molluscs, annelids, arthropods and mammals, makes it likely that these peculiar bodies are universal in flagellate spermatogenesis, and probably have their counterpart in sperms such as that of *Astacus*.

Methods.

Three standard methods for mammalian spermatogenesis were used for this work. The Kolatchev, Champy-post osmication, the Regaud, chilled formol bichromate hæmatoxylin, and the chilled Da Fano, cobalt nitrate formalin and silver method. The figures given on Plates 28-29, figs. 1-12, refer to Da Fano material alone.

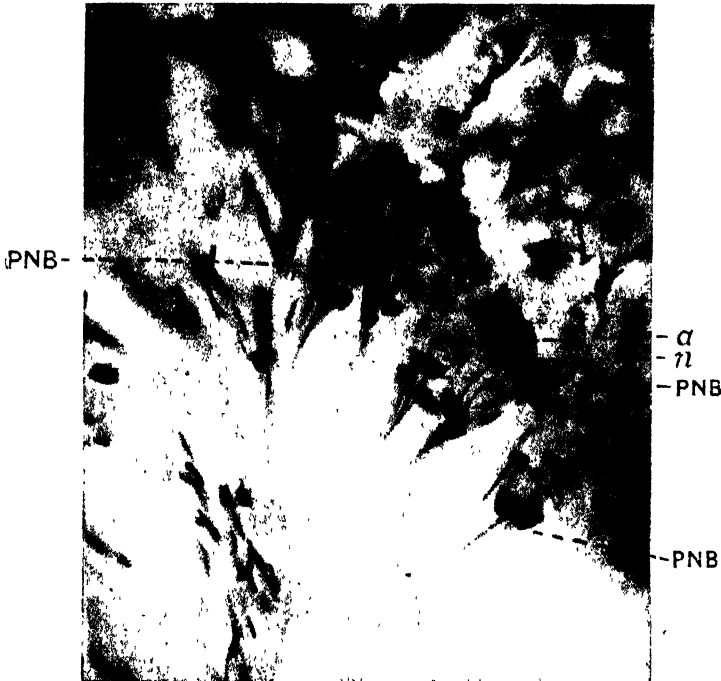
The usual procedure was to place capsules containing the fixatives in ice

and salt in a dish till the fluids had reached 5 to 6° C. The guinea-pig, stunned by a blow on the head, had its throat cut, and the testis was cut out immediately. Quite small pieces were placed in the fixatives, which were left in the dish (till the ice had all melted) for the usual fixation periods. There can be no question that the chilling of the fixative has a very important effect on the plumpness of the cells seen in the finished sections, that shrinkage, usually the bugbear of the formalin-Golgi methods, is much lessened, and the resultant preparation provides something more than a mere demonstration of the Golgi apparatus.

The figures on Plate 30 are drawn with the camera lucida, and are not improved for publication purposes. The Da Fano slides were counterstained for a short time in Mann's methyl-blue eosin.

Original Observations.

The Ripe Spermatozoon. In the accompanying text-fig. is a microphoto of part of a spermatic tubule, from a chilled Da Fano preparation. The post-nuclear bands are quite clearly shown at PNB. In Plate 29, figs. 7 and 8,



Microphotograph of part of spermatic tubule of *Cavia*, prepared by the chilled Da Fano method, showing ripe spermatozoa with post-nuclear bands (PNB), acrosome (α) and nucleus (n).

are two ripe spermatozoa, by the same method, counterstained in Mann's methyl-blue eosin. At *a* are the parts of the acrosome, *n* is the nucleus, which is partly covered by a dark, usually chocolate band (PNB) forming a capsule in which the nucleus fits. The middle piece Golgi granule is at *Y*, the middle piece (mitochondrial) at *m*.

These bands are very remarkable objects, and the writers have never seen anything quite like them before in mammals. Retzius, of course, in his beautiful studies on mammalian spermatozoa, often draws the confines of the bands as a grey section of the sperm head, but no method except Da Fano picks out these bands in such an extraordinary manner. With Da Fano's fixative the bands are extremely argentophil and are the most noticeable cytological feature of the testis.

The Spermatocyte.—In Plate 28, fig. 4, is a full grown spermatocyte showing the Golgi apparatus at *g*, and in the cell, another structure at PNG. In figs. 5 and 6 are two other examples of the Golgi apparatus with the same type of structure attached. This is the normal position, and in very well-fixed and impregnated cells, the granules form the cortex of a vacuole. In fig. 6 the vacuole has collapsed, and as a result the granules are thrown together. This is a very common appearance.

Now there can be no doubt whatever that the structure shown at PNG in these figures is not the vacuolar system. The latter is formed of more numerous granules and occupies a different part of the cell. On the other hand there is a now well-known argentophil structure on the middle-piece (*Y* in figs. 7, 8, 11 and 12) which was discovered by Perroncito, and has been studied by Weigl, the senior writer and Woodger, and more recently by Brambell and Subba Rau, and which might possibly be the structure marked PNG in figs. 4, 5 and 6.

It is not possible to say definitely that this very clearly marked body in the spermatocyte is not the forerunner of the argentophil body of the middle-piece. There are two reasons why this interpretation is doubtful. First, the argentophil bead of the middle piece has been traced by four observers to a bleb extruded from the spermatid Golgi apparatus; secondly, a beaded formation of the middle-piece is rarely seen. After prolonged study of this question we have not succeeded in offering a more satisfactory evidence than this.

The Spermatid.—There can be no doubt about the presence of the three structures in the spermatid. On the plates, figs. 1, 2, 3, 9, 10, 11, 12 are spermatids in various stages of development. The cell drawn in fig. 1 is the earliest, the proacrosomic material (*a*) still being inside the Golgi apparatus (*g*). The post-

nuclear granules have become coarser, and are grouped behind the nucleus, at PNG. The peculiar bead, which is almost certainly the forerunner of the so-called middle-piece Golgi apparatus, is shown at Y. A later stage is depicted in figs. 9 and 10. The acrosome has become plastered upon the nucleus, and there has appeared at *s* a space at each side of the nucleus. This is apparently formed by liquid which is being squeezed out of the nucleus as the latter contracts to form the sperm head. The post-nuclear granules are as before, though in fig. 9 they are not so flattened upon the nucleus.

In figs. 2 and 3 are subsequent stages. In the first of these figures the granules form a halo around the hinder part of the nucleus, and in the next figure the arrangement is even more definite.

Careful examination of many of these stages shows that the granules are often elongate hollow structures, which seem to expand till they touch, and finally unite to form the solid covering which is shown in fig. 12. This expands further till it forms the cuplike structure depicted in the spermatozoa shown in figs. 7 and 8. This finding is certainly the correct one, and is quite easily established.

The spermatid in fig. 11 had been treated with X-rays, and the band is in an unusual position. This subject will be dealt with later, in another communication.

Post-nuclear Bodies in other Animals.

Amphibian Spermatogenesis.—Amphibian spermatogenesis has been studied by a number of well-known cytologists, *e.g.*, Hermann, McGregor, Meves, Bowen, etc. The work of McGregor on *Amphiuma*, and that of Bowen on *Plethodon* alone will be considered here. In Plate 30, figs. 37 and 38, are two stages after McGregor from E. B. Wilson. The centrosome (C), in fig. 37, is accompanied by a ring (R), which has been well described by Hermann and Meves; at the hinder end of the nucleus is a large body (PNB) which we consider a post-nuclear body. In fig. 38, a riper spermatid is shown, the same parts being lettered as before.

Turning now to Bowen's *Plethodon* shown in figs. 13 to 18, we find that he draws a diploid body attached to the outgrowing flagellum in fig. 13. We interpret the first of these as the post-nuclear granule, and the second as the centrosome. In figs. 14 and 15, the centrosome, we presume, has become embedded in the rapidly swelling post-nuclear body, which in the subsequent figs. 16 to 18, forms the same parts as shown already from McGregor in *Amphiuma* (fig. 38).

The nature of the remarkable ring is unknown. We suggest that it is mitochondrial, though it may be the other half of the divided centrosome.*

Insecta.—Bowen has given a clearly illustrated account of the spermatogenesis of the moth, so far as concerns the bodies herein discussed.

In Plate 30, figs. 25A and 25B, are two advanced spermatids showing at PNG, the body marked B, in Bowen's paper and called by him, "nuclear body of doubtful nature." In Bowen's figs. 25, 32, 35, 39, 48, 50, 52, 53, 56 and 57, it seems established that this body is not inside the nucleus, but on the nuclear membrane. In his figs. 48, 49, 50, 52, 53, 56 and 57 it is established that the "nuclear body of doubtful nature" bears a close topographical relationship to the centrosome-cum-flagellum, as is shown in his two figures reproduced in this present paper (figs. 25A and 25B, marked in Bowen's paper figs. 52 and 56).

Now we consider that in fig. 25B the three structures, centrosome (*c*), acrosome (*A*), and post-nuclear granule (PNG, Bowen's *B*), are clearly shown. In fig. 25A the centrosome has come into direct contact with the body PNG, and cannot be distinguished. In any case it seems certain that the centrosome becomes either elongated, so much like the flagellum as not to be distinguishable from the latter, or that it ceases at this period to stain as a separate granule. In fig. 25B (Bowen's fig. 52) the nomenclature except for *B* (our PNG) is the same as Bowen's.

Now fig. 25B is very important because, as will be seen later, it provides quite clearly the key to the explanation of the *Lepisma* spermateleosis, which appears to us to have been confused by both Bowen and Charlton.

In subsequent stages (Bowen's figs. 57 to 60) the relationship between acrosome, centrosome and post-nuclear apparatus is not interrupted. That is to say, there is a filament on the lepidopterus sperm head, which passes forwards

* Recently Dr. E. S. Goodrich has sent us some axolotl testis slides from the Oxford Collection. Unfortunately, these were prepared by acetic corrosive alcohol, but they show the centrosomes well. The body marked PNG in figs. 13-18, and in fig. 38, my pupil R. N. Mukerji and I find to be the head centrosome. The ring (*R*) is budded off in the early spermatid, and is the corresponding moiety of the head centrosome. We feel that there can be no doubt as to this interpretation, as the cells are so large and clear. The third granule, unlabelled in figs. 15-17, is, we now consider, the real post-nuclear body, but it is dissolved away in corrosive alcohol acetic. The lettering given in Pl. 30, fig. 37, for McGregor's *Amphiuma* is probably correct, but until we have had the opportunity of studying suitably fixed urodele testis, it is useless to speculate further; it should be noticed, however, that in the *Amphibia* studied there are three bodies in the post-nuclear region of the spermatid, one of which is certainly not a centrosome. (March 14.)

as the acrosome and which passes backwards into elongated post-nuclear body, and then into centrosome and flagellum behind. We turn for a moment to the pentatomid Murgantia. In Plate 30, figs. 26 to 28, are three of Bowen's examples. At PNB is Bowen's "pseudo-blepharoplast," which Bowen has clearly demonstrated as separate from the centrosome or centrosomes; this is the post-nuclear body. Speaking of this Bowen says "this chromatic body seems to be dissolved into the nuclear sap, being often surrounded by a clear vacuole during the process of dissolution. In *Euschistus*, Montgomery described a movement of this body into the nuclear cavity with a subsequent disappearance, but his interpretation was incorrect because he believed the body to be a centriole."

Bowen's description of the disappearance of the post-nuclear body after granulation seems somewhat doubtful to us

In the beetle *Cincindela*, Bowen has figured a "chromatic plate" (Plate 30, fig. 25, of this present paper) which was previously noted by Goldsmith and called the "middle-piece" or "neck plate." Bowen writes, "if, as is probable, the chromatin be concentrated on the inner surface of the nuclear wall, the flattened basal area will lead to development of an apparent chromatic plate The optical reasons for this appearance are too well known to require further comment." Bowen seems to have mistaken the post-nuclear plate for an aggregation of chromatin. The view of Goldsmith is, we consider, the more correct of the two; it is really more in the nature of a "middle-piece" than a chromatinic mass.

It seems that in *Lepisma* Bowen may have misunderstood the true nature of the post-nuclear apparatus and this has led him into an error. Bowen writes (1) "According to Charlton, the centriole, a very conspicuous body, becomes applied to the anterior tip of the sperm head, as the acrosome—a relationship not new in cytological literature, but now discredited by the results of research completed since Charlton's work was done. This led me to suspect that Charlton's account must be fundamentally mistaken, though in just what way I was unable to guess. With the clearing up of the *Cincindela* case, however, it became clear to me that in *Lepisma* the centriole probably did have the history assigned to it by Charlton, but was not the acrosome, and was in no way related to it. A re-examination of Charlton's paper revealed the presence of another previously troublesome body which he had called the "middle-piece, from its being located just posterior to the head. From the history and staining reactions of this 'middle-piece,' it occurred to me that it might be the acrosome—an interpretation rather

damaging to the usually accepted views of the function of acrosomes, but offering a possible way out of the more serious difficulties involved in Charlton's explanation."

In Plate 30, figs. 19 to 24, are copies of some of Bowen's figures of *Lepisma*. The normal spermatid in fig. 19 gives rise to a normal elongate spermatid as in figs. 20 and 21, with, in the latter figure, a post-nuclear granule at PNG, not found in the cell depicted in fig. 20, perhaps hidden by the Golgi apparatus (*g*). In fig. 22, probably all the elements in question are figured, A, the acrosome, PNG the post-nuclear granule, and *c* the centrosome. Compare now Bowen's figure 25B (in this paper) with fig. 21. In the former (moth) we have already discussed the various parts, and we have pointed out that excepting for Bowen's identification of his body B (our PNG) as "nuclear body of doubtful nature," our two accounts of this spermateleosis are in agreement. Now the body marked A, in fig. 21, is called centrosome by Bowen and Charlton. It is, we consider, the same structure as shown in fig. 25B, and is really acrosome, passing back into centrosome and flagellum behind. As shown by Bowen in his original figs. 52 to 60 there is a connection between the clearly marked acrosome and the poorly defined centrosome and flagellum behind. Fig. 24 of this paper corresponds completely to fig. 57 of Bowen's paper. As Bowen shows in his picture of the *Lepisma* spermatid head in fig. 23 of this paper, there is a connection between the acrosome A (his centrosome) and the post-nuclear body, PNG (his acrosome). In fig. 24, the post-nuclear body resembles closely that of the amphibian (fig. 37), but as Bowen shows, in *Lepisma* it elongates much further.

Mollusca.—The post-nuclear granules are probably the largest in the slugs (*Arion* and *Limax*) where the senior writer had no trouble in finding them in the young spermatocyte still in the early prophase of the heterotypic division (Plate 30, fig. 29). In the early spermatid (fig. 30) they form a rounded group, which later (fig. 31) passes to the back of the nucleus, forms a plate, and ultimately fuses up with the nucleus (figs. 32 and 33). Many post-nuclear bodies are figured in the senior author's study on the germ cells of *Limax*, *Arion*, *Testacella* and *Helix* (3).

Annelida.—In *Saccocirrus* three post-nuclear granules occur (figs. 34 and 35). In fig. 36 the end view of the spermatid is shown, with the post-nuclear wedges between the mitochondrial spheres (M).

Relationship between Centrosome and Post-nuclear Plate.

In the mollusc (Plate 30, fig. 33) and insect (figs. 26 to 28, etc.) the centrosome passes through the middle of the post-nuclear plate, into the hinder part of the nucleus. The post-nuclear plate serves as a sort of supporting disc fitting the centrosome and flagellum into the nucleus. In other animals, such as the amphibian (fig. 37), this relationship does not hold good, and the post-nuclear body is never a plate, but either spherical or elongate. In mammals the post-nuclear body forms a wineglass-shaped structure into which the nucleus sits. The centrosome complex comes into contact with the bottom of the post-nuclear apparatus.

From these observations it seems likely that in some sperms the centrosome is normally embedded in or in contact with the post-nuclear body, which itself forms a supporting or uniting contrivance between nucleus and flagellum.

Summary.

Post-nuclear bodies are known in mammals, amphibians, annelids, molluscs and insects. They are probably universal in flagellate spermatozoa.

Their function appears to be to provide a supporting or uniting contrivance between the attenuated sperm head and the vibratile tail.

The formation of the post-nuclear body in the spermatogenesis of *Cavia* is followed out.

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DESCRIPTION OF PLATES.

Lettering.—*a*, A, acrosome. *c*, centrosome. *g*, Golgi apparatus. *M*, *MP*, mitochondria and middle-piece. *N*, nucleus. *PNB*, *PNG*, post-nuclear system. *R*, centrosome ring. *S*, space around nucleus. *V*, vacuolar system. *Y*, Golgi middle-piece bead.

PLATE 28.

FIGS. 1, 2, 3.—Three stages in spermateleosis of *Cavia*, showing grouping of post-nuclear granules, *PNG*.

FIGS. 4, 5, 6.—Spermatocyte of *Cavia*, showing in two latter figures near the Golgi apparatus, the supposed post-nuclear primordia, *PNG*.

PLATE 29.

FIGS. 7, 8.—Ripe *Cavia* sperms, by Da Fano's method.

FIGS. 9 and 10.—Two early spermatids showing post-nuclear granules, *PNG*.

FIGS. 11 and 12.—Later spermatids.

PLATE 30.

FIGS. 13 to 18.—*Plethodon cinereus*, after Bowen.

FIGS. 19 to 24.—*Lepisma domestica*, after Bowen.

FIG. 25.—*Cincindela sex guttata*, after Bowen.

FIGS. 25A and 25B.—*Callosamia promethea*, after Bowen.

FIG. 26.—*Brochymena quadripustulata*, after Bowen.

FIGS. 27 and 28.—*Murgantia histrionica*, after Bowen.

FIGS. 29 and 30.—*Arion hortensis*, after Gatenby.

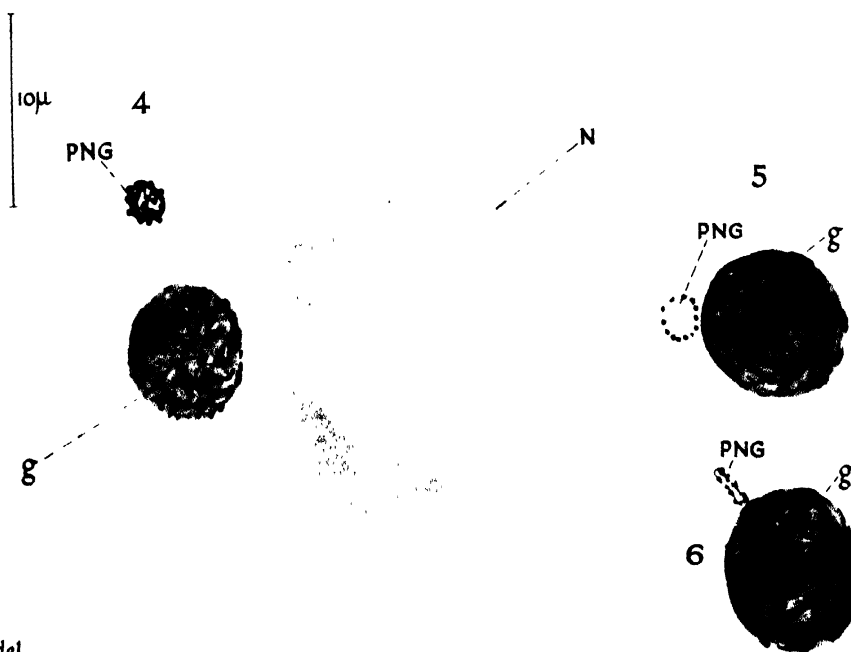
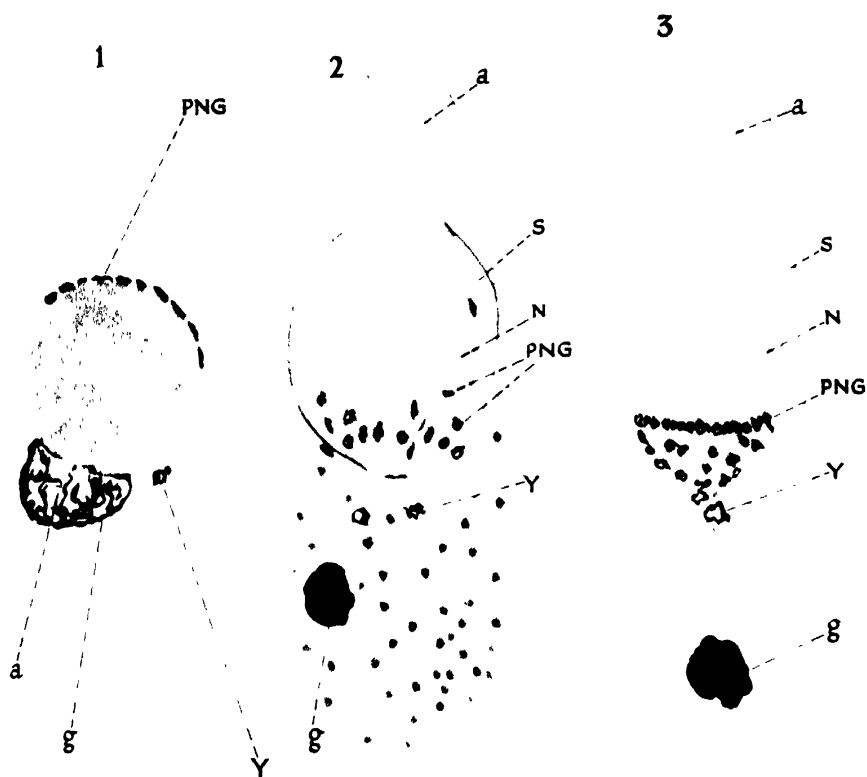
FIG. 31.—*Testacella*, after Gatenby.

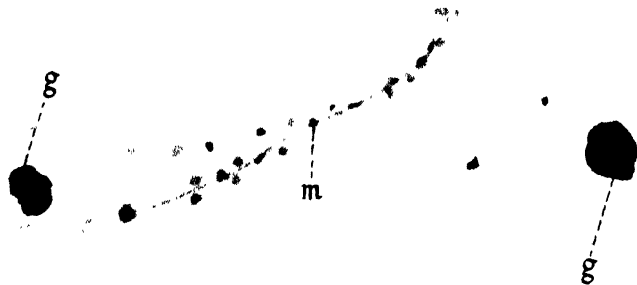
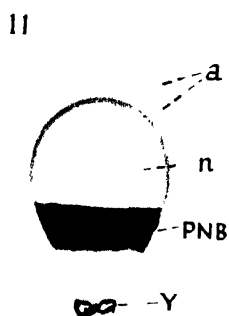
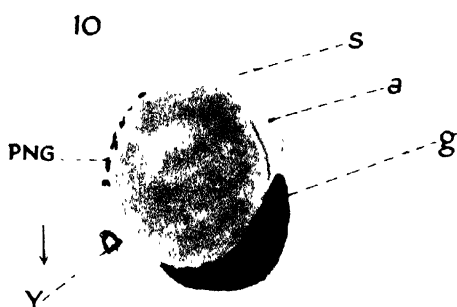
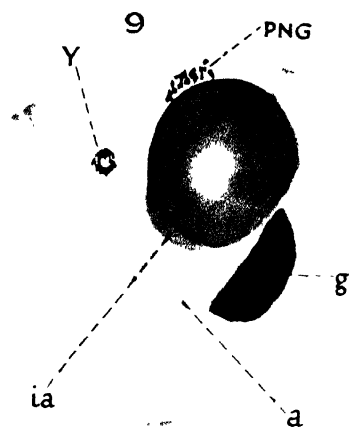
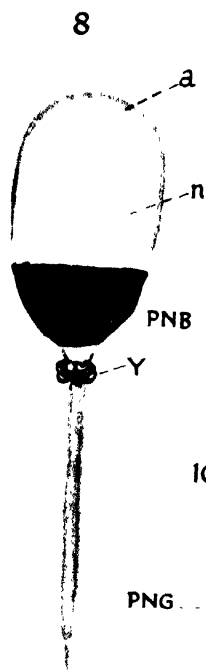
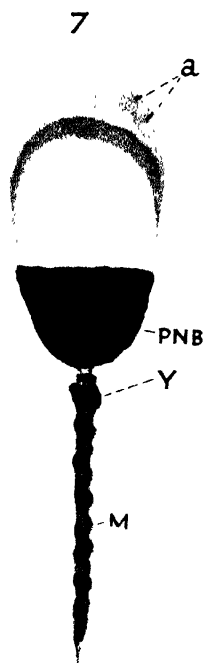
FIG. 32.—*Arion ater*, after Gatenby.

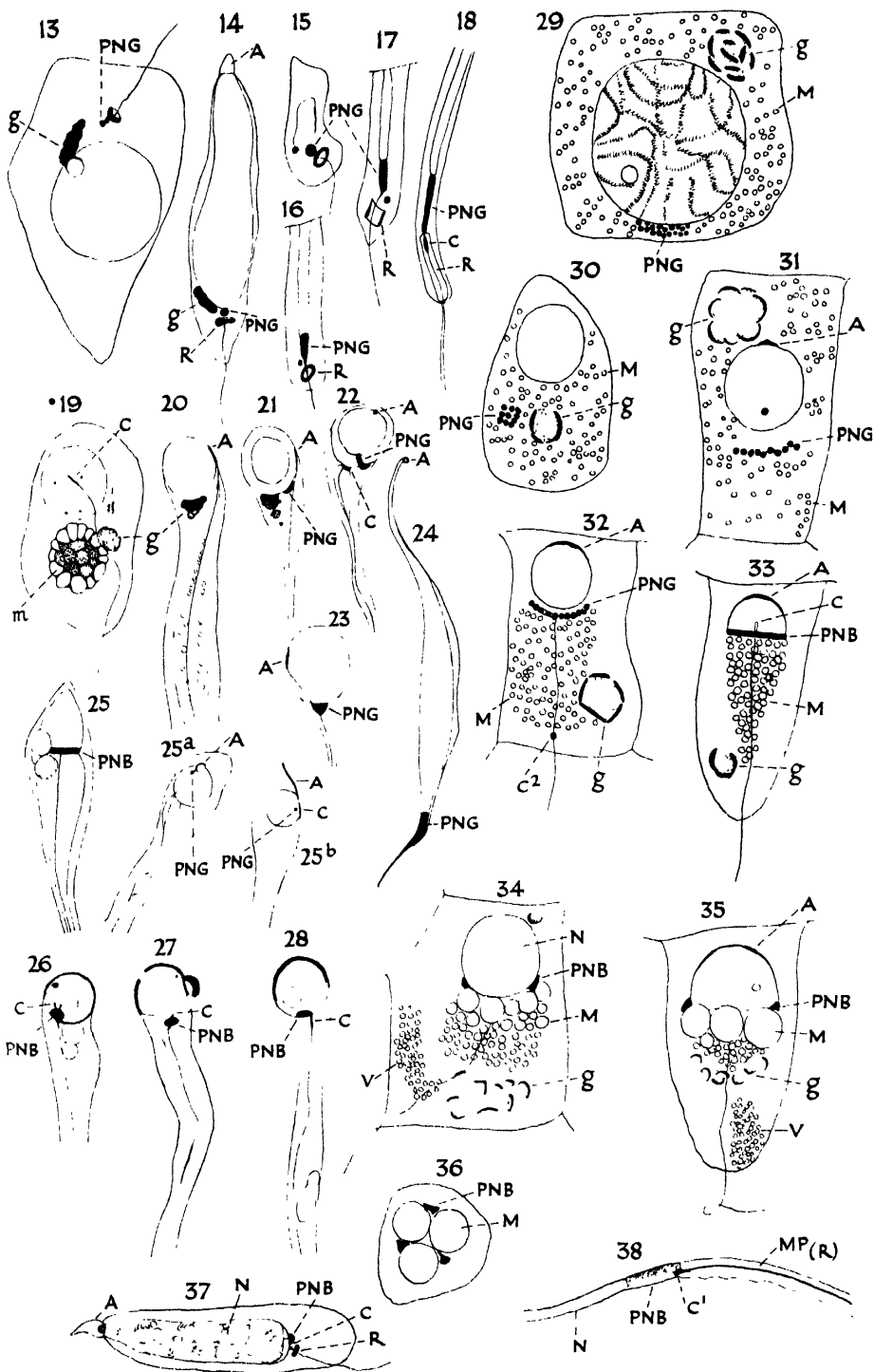
FIG. 33.—*Limax agrestis*, after Gatenby.

FIGS. 34, 35 and 36.—*Saccocirrus*, after Gatenby.

FIGS. 37 and 38.—*Amphiuma*, after McGregor.







The Rôle of the Young Lucerne Plant in Determining the Infection of the Root by the Nodule-forming Bacteria.

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(Communicated by Sir John Russell, F.R.S. - Received February 11, 1929.)

[PLATE 31.]

Observations have been made at Rothamsted over a period of about five years upon the development of nodules on young seedlings of lucerne (*Medicago sativa*, L.). Some thousands of seedlings have been examined in various experiments and it was found to be the rule that the first appearance of nodules coincided with that of the expansion of the first true leaf. When lucerne is sown under summer glasshouse conditions, in pots of soil or sand, the seedlings are up in from 3 to 5 days, and in 6 to 8 days the first true leaf becomes visible. This is at first closed, but in 8 to 12 days from the date of sowing it opens out (fig. 1).

The following experiment illustrates the relationship between the opening

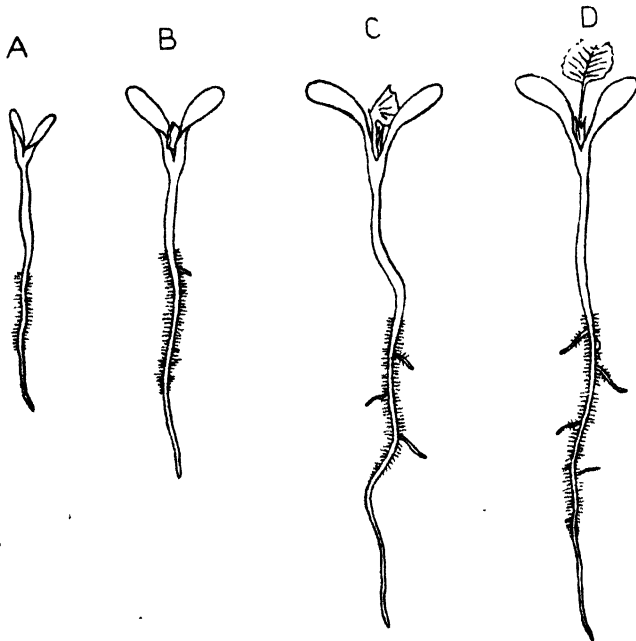


FIG. 1.

of the first leaf and the appearance of nodules. Lucerne seed, inoculated with nodule bacteria was sown in 12 pots, each containing about 8 pounds of sand, and these were watered with a plant-culture solution free from nitrogen.* Five days after sowing the seedlings were up, and in another 4 the first true leaf, still closed, could be seen on most of them. The appearance of nodules and the opening of the first true leaves is shown in fig. 2, where each point represents observations made upon 20 seedlings, 10 from each of duplicate pots. The general agreement in the time of appearance of nodules and in the opening of the first true leaves is evident.

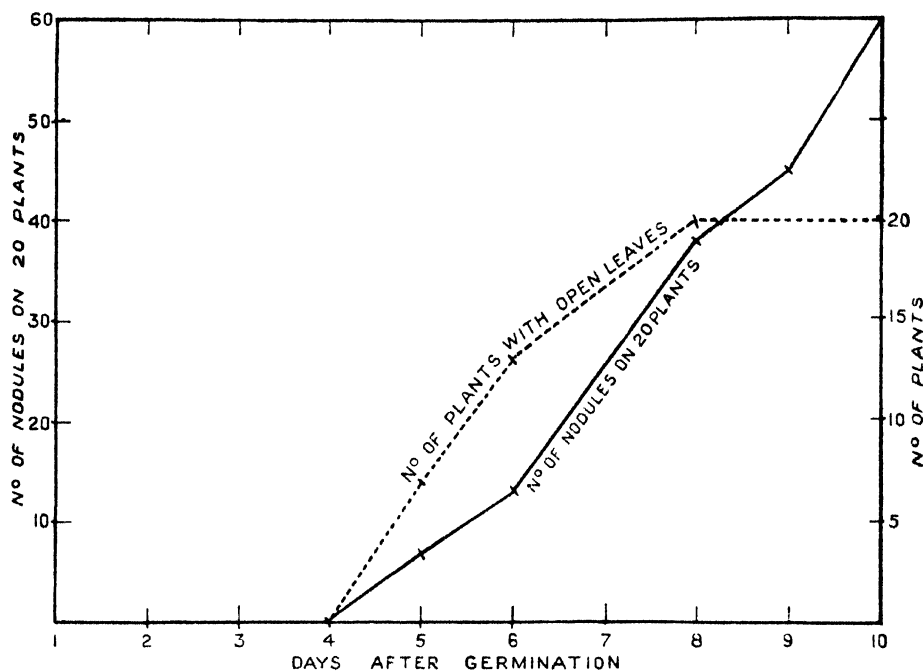


FIG. 2.

The closeness of this agreement can be more clearly appreciated when, instead of making such counts from samples of a seedling population, the course of development and nodule appearance is followed on the same individual plants grown in water culture. For this purpose seedlings were grown in test-tubes containing the food solution referred to above. Observations were made at intervals on the same 20 seedlings. The appearance of nodules is

* The plant-culture solution used throughout this work had the following composition:—Water, 1000 c.c.; KCl, 0.74 grs.; K_2HPO_4 , 0.3 grs.; KH_2PO_4 , 0.3 grs.; $MgSO_4 \cdot 7H_2O$, 0.5 grs.; NaCl, 0.5 grs.; $CaSO_4$, 0.5 grs.; $FeCl_3$, 0.04 grs.

shown in fig. 3, where the age of each seedling is taken from the date of the opening of the first true leaf. Only one nodule had appeared on the day before this opening, although the roots had been in contact with a suspension of the bacteria for 9 days. Four more nodules appeared on plants the same day that the leaf opened and 8 more the following day.

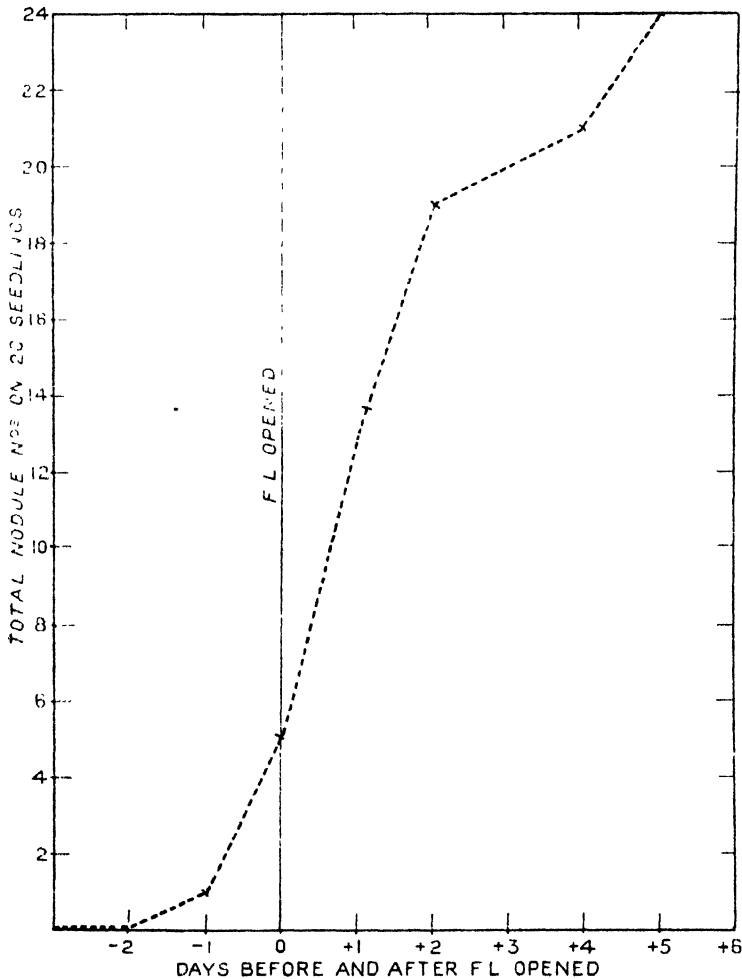


FIG. 3.

The appearance of nodules in another similar set of water cultures is shown in fig. 4, where the appearance of lateral roots and of the first leaf still in a closed condition are also recorded. In view of the comparisons sometimes drawn between nodules and lateral roots—it is interesting to note that the time of first appearance of the two do not coincide. The experiment also shows

that appearance of the first nodules corresponds with the *opening* of the first leaf and not with its emergence from the growing point.

In the last set of cultures the roots had been in contact with a suspension of nodule bacteria for 10 days before the opening of the first leaves and the

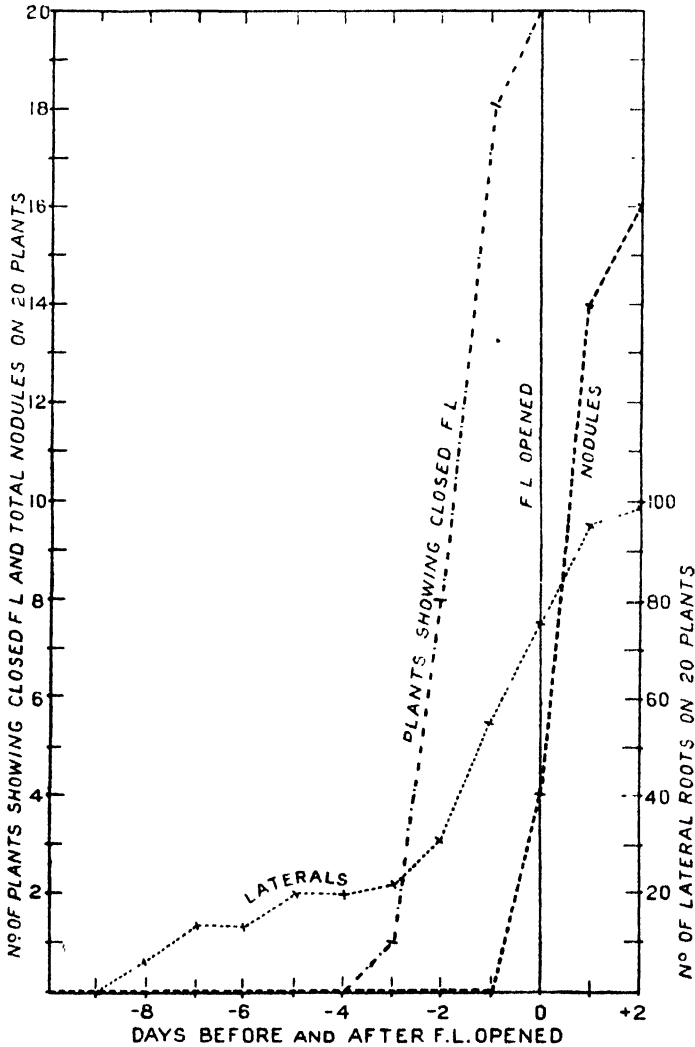


FIG. 4.

appearance of nodules. In seeking an explanation of this lag it seemed necessary to discover first whether the organisms fail to enter the root hairs during the "cotyledon stage" or whether, having entered the plant, they are unable to induce the formation of a nodule. Owing to the transparency of the root

cortex, it is possible to see a nodule in the very early stages of its growth. When a root hair has been infected the infection strand passes through the cortical cells as far as the endodermis and, along its course, the cortical cells become more densely protoplasmic and commence to divide (fig. 5). In such a very young stage the cells affected are only about 25 in number but their increased opacity renders the "nodule" clearly visible in the living root. The absence of visible nodules thus implies that this earliest stage has not even been reached.

It was therefore a question whether the organism enters the root during the

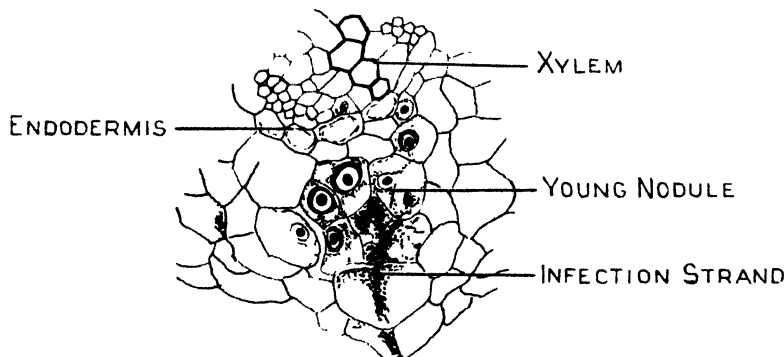


FIG. 5.

cotyledon stage or whether it is then normally unable to infect the root hairs. To determine this, seedlings were grown aseptically in boiling tubes containing a plant-food solution in agar. Twelve seeds, the coats of which had been sterilised by immersion in absolute alcohol followed by 0.2 per cent. HgCl_2 washed off with sterile water, were sown in each of 4 tubes. When they had germinated, a suspension of the nodule organism was introduced and the roots were examined at daily intervals, a search being made for infected root hairs. Within 4 days of germination most of the plants showed the first leaf in a closed state. Four days later the majority had the first leaf open. During the 8 days that thus elapsed between inoculation and the opening of the first leaf the roots were surrounded by large numbers of organisms, many in a motile state, but not a single root hair containing an infection thread was seen. The absence of the bacteria other than the nodule organism was shown by plating at the end of the experiment. One day after the opening of the first leaf on the majority of plants about 2 per cent. of the root hairs contained infection threads, which were easily seen owing to their high refractive index.*

* Previous observations on slightly more advanced seedlings showed 4 per cent. of the hairs with infection threads (Thornton (1)). Pierce (2) however, claims to have obtained a much heavier infection on roots placed between layers of filter paper.

The evidence thus indicates that the bacteria do not normally enter the root before the first leaf opens.

This delay in the infection of the root until the opening of the first leaf may be wholly or partially due to some factor in the plant's physiology or may represent a period of development of the nodule organisms outside the plant necessary to enable them to reach an infective condition (*cf.* Thornton and Gangulee (3)). If the delay were solely due to the organisms, the time taken for nodules to appear should be independent of the age of the seedlings. The following experiment was therefore made to determine how soon after their addition to the sand the nodule bacteria can produce nodules on older, as well as on the young seedlings.

Lucerne seed was sown in 10 pots of sand sterilised with steam and watered with sterile plant-food solution. The seed coats were sterilised as described above and 2 pots (Series A) were left uninoculated to serve as controls against contamination. Four pots (Series B) were sown with uninoculated seed and, after 3 weeks' growth, when the seedlings had a widely expanded first leaf, these pots were inoculated by pouring on a thick suspension from a young culture of the nodule organism. Twenty seedlings from this series were examined 3, 5, 7 and 9 days after inoculation. The mean number of nodules per plant on each occasion is shown in fig. 6. A considerable number of nodules had appeared on the fifth day after inoculation.

That these nodules were produced by the culture added was shown by the almost complete absence of nodules on the control plants, examined at the end of the trial. On the day on which Series B was inoculated another 4 pots (Series C) were sown with seed and similarly inoculated. Seven days later the first leaves of the series were showing but had not opened. On the seventh, ninth, eleventh and thirteenth days after sowing and inoculation 20 seedlings from these pots were examined. The course of nodule appearance is shown in fig. 6. The appearance of the nodules was delayed until the true leaves opened after 9 days although Series B showed that the culture was capable of producing nodules within 5 days from the date of inoculation. The delayed appearance of nodules must therefore be due to the plants.

The rule that no nodules appear before the expansion of the true leaves is not absolute. When a large number of seedlings are grown together in sand culture there is a period during which the first leaf has opened on some plants but not on others. At this time nodules appear on the former plants but also on a few of the plants whose first leaves have not yet opened. Thus in the pot experiment illustrated in fig. 2 the examination made on the fifth day

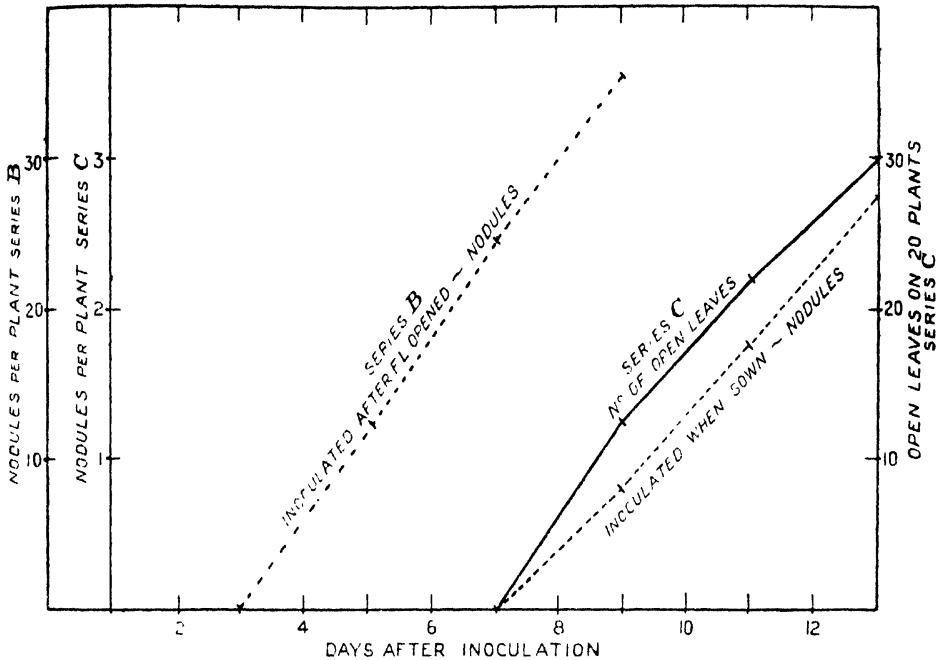


Fig. 6.

after germination showed the following distribution of nodule-bearing plants :

Nodule-bearing plants, with leaf open	5
" " without open leaf	2
Plants without nodules, with leaf open	3
" " without open leaf	10

Other examples are shown in the control series shown in Tables I and II. In normal cases only a small proportion of such younger plants bear nodules. In one pot experiment, however, a wholly abnormal percentage of nodules appeared on plants whose first leaf had not yet opened. In this experiment the inoculated seed had been sown thickly in sand watered with plant-food solution. The seed germinated unevenly, some plants showing open leaves after 9 days, while 12 days after sowing only about half the plants had the first leaf open. On the twelfth day 20 seedlings with open leaves and 20 with leaves still closed were taken at random from 4 pots and examined. The former had a total of 96 nodules while the latter had 64.

This experiment was abnormal in that, owing to uneven germination, young plants with the first leaf unopen grew for some days mingled with older plants

in which the leaf had expanded. An experiment was therefore made in which this condition was deliberately reproduced. Four pots of sterilised sand, watered with sterile plant-food solution, were prepared, and in two of these lucerne seed sterilised as above described was sown thinly. Fourteen days later the seedlings bore large expanded first leaves. Fresh seed was then sown in these pots among the seedlings and at the same time the two remaining pots were sown and all four pots inoculated with a suspension of the nodule bacteria. After 10 days the later sown seedlings showed the first leaf still closed. Fifty of these younger seedlings from each pot were examined with the result shown in Table I.

Table I.—Effect of the Presence of Older Seedlings upon the appearance of Nodules on Young Seedlings.

	Number of nodules on 50 seedlings with the first leaf closed.		
	Plot 1.	Plot 2.	Mean.
Only young seedlings present	6	5	5.5
Young seedlings growing amongst older	38	35	36.5

The presence of the older plants had induced the formation of a considerable number of nodules on the young plants growing amongst them at the stage when only occasional nodules would normally have appeared. This influence of the older plants upon their surroundings suggests the extrusion of some substance from the roots. To investigate this possibility an attempt was made to extract the solution from sand cultures in which lucerne seedlings had been growing. Pyrex glass troughs, 10 × 5 inches in area and 2½ inches deep, were filled with sand watered with equal volumes of plant-food solution and sown thickly with lucerne seed. When the seedlings were in the cotyledon stage the contents of one trough was carefully packed into a glass tube, 2 inches in diameter and 3 feet 6 inches long, clamped in a vertical position and fitted at the lower extremity with a perforated rubber bung and a glass delivery tube. The solution was extracted with the pressure of a head of distilled water, about 1 litre of the solution being thus obtained (Extract A). When the seedlings in the second trough had well-expanded first leaves the solution from this culture was extracted in a similar manner (Extract B). The two extracts were sterilised in the autoclave. Their action was tested in the following manner.

Six pots were filled with sand, sown with lucerne seed and watered with plant-food solution. When the seedlings were up 2 pots were inoculated with a suspension of the nodule bacteria in plant-food solution made up in distilled water, 2 pots were inoculated with a suspension in plant-food solution made up in Extract A and 2 pots with a suspension in a similar solution made with Extract B. 15° c.c. of each solution were thus added per pot. Ten days after sowing some of the seedlings showed first leaves just open. Twenty plants with the leaves open and 20 with them still closed were examined from each pot, with the results shown in Table II.

Table II. --Effect of the Solution Surrounding the Roots.

	Number of nodules upon 20 plants.					
	Plants with first leaf open.			Plants with first leaf closed.		
	Pot 1.	Pot 2.	Mean.	Pot 1.	Pot 2.	Mean.
Watered with food solution alone ..	25	34	29.5	0	5	2.5
Watered with food solution + Extract A	20	20	20.0	0	7	3.5
Watered with food solution + Extract B	32	34	33.0	18	13	15.5

The extracts have produced no effect on the nodule numbers on such plants as had the leaves already open. The Extract A produced no significant effect at all, but Extract B, prepared from sand in which older seedlings were growing, caused a marked development of nodules on seedlings whose first leaf had not yet opened, increasing their numbers six-fold as compared with the control plants in the same stage. It would thus appear that, when the lucerne seedlings have attained a stage of development marked by the opening of the first true leaf, some substance is extruded from the roots which stimulates the infection of the root hairs by the nodule bacteria.

In order to observe whether the extracts had any influence upon the growth of the nodule organism, the plant-food solution plus 1.5 per cent. agar was made up with distilled water, with Extract A and with Extract B. A similar set of media were made up containing 1 per cent. saccharose. The media were sterilised in the autoclave, poured into sterile petri dishes and streaked with a 2-day old culture of the lucerne nodule organism. After one week's incubation

at 25° C. scarcely any growth appeared on the media without saccharose. In the presence of saccharose no growth showed on the medium made up with distilled water, a slight opaque growth on the medium made up with Extract A and a considerable slimy growth on the medium containing Extract B. (See Plate 31.)

The substance formed by the older seedlings thus has an effect upon the growth of the nodule bacteria, but this effect would seem not to consist in supplying energy material since it showed itself only when sugar was added. The extracts did not reduce Fehling's solution and tests for sucrose and for pentoses were also negative. A pot experiment was made in order to see whether the presence of an amino acid would induce nodule formation on seedlings before the first leaves opened. Inoculated seedlings were given food solution alone and food solution containing 0·01 per cent. and 1·0 per cent. asparagine. In neither case were the nodule numbers increased by asparagine.

The manner in which the extract stimulates infection is not at present clear. Its effect in increasing the number of bacteria may be of some importance but observations of root hairs in plants grown on agar, referred to above, tend to show that this cannot be a limiting factor since seedlings in the cotyledon stage do not show infected root hairs even in the presence of considerable numbers of the bacteria. It is observable that in this case the bacteria exist either as individuals or else in loose clumps among the root hairs, whereas the infection of a root hair is preceded by the formation of a minute colony of bacteria imbedded in slime, showing as a bright refractile spot on the wall of the root hair. Such spots are illustrated by Marshall Ward (4). The fact that a slimy growth of the bacteria was induced when these were growing on agar containing Extract B may therefore be relevant.

The coincidence in time of nodule appearance with the opening of the first leaf raises the question as to whether these two events are causally connected or whether they are both the effect of some other physiological change in the seedling. To answer this question the course of nodule development was followed on seedlings from which the true leaves were removed. Lucerne seedlings were grown in test-tubes containing plant-food solution inoculated with a suspension of the nodule bacteria. In one series the true leaves were cut off as soon as they appeared; in the second series the cotyledons were cut off but the true leaves allowed to develop, and in the third, control series, the true leaves and cotyledons were left. The course of nodule development is shown in fig. 7.

On the control plants the appearance of the nodules followed the opening

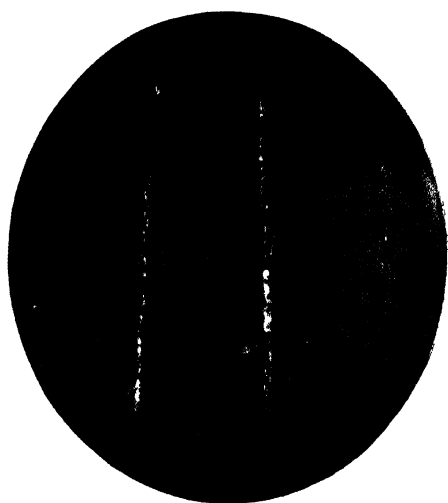


FIG. 1. Lucerne Nodule Bacteria, growing on Agar with Nutrient Salts and Saccharose.

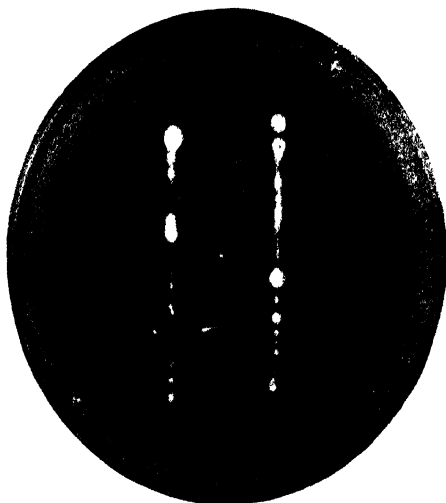


FIG. 2.—Lucerne Nodule Bacteria, growing on Agar with Nutrient Salts, Saccharose and Extract A.



FIG. 3.—Lucerne Nodule Bacteria, growing on Agar with Nutrient Salts, Saccharose and Extract B.

of the true leaves. In the series with the cotyledons cut, the opening of the true leaves followed a course similar to that shown for the control plants, but

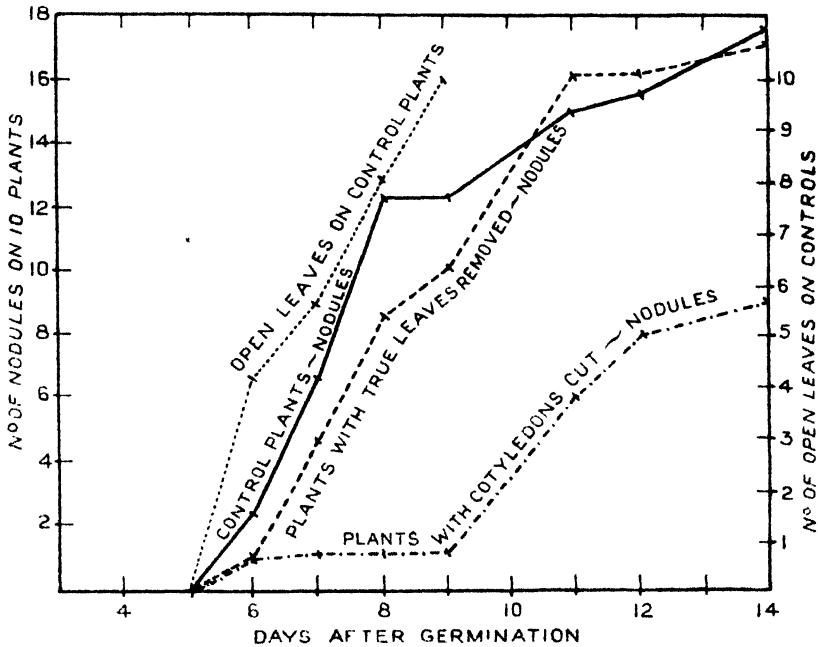


Fig. 7.

the development of nodules was delayed until considerable growth of the true leaves had occurred. This indicates that the passage into the root of substances from the cotyledons is one of the factors controlling the early appearance of nodules. Where the true leaves were removed, however, the normal course of nodule appearance was not significantly altered.

The substance affecting nodule formation is therefore not produced in the true leaf at the time of its expansion since its action is unaffected by the removal of the leaf. It seemed possible that it is formed in the growing point of the top. In the hope of testing this possibility, a sand culture experiment was made, in which seedlings were grown in three series of duplicate pots, those in the first series having the leaves cut before expansion, those in the second series having the terminal bud cut out and those in the third control series being untouched. Three weeks after sowing the control plants had one expanded leaf and, in the majority, a second closed leaf just appearing. Fifteen plants from one pot of each series were then washed and their nodules counted. Twenty days later a second similar examination was made. There were no significant

differences in nodule numbers between any of the series. The removal of the terminal bud therefore did not affect the number of nodules formed. It was found, however, that in most of the plants two fresh buds were regenerated at the bases of the cotyledons, so that this series merely showed that severe damage to the shoot meristem does not affect the formation of nodules. The results from the first series, on the other hand, confirm the previous experiment in showing that the appearance of nodules is not checked by removal of the first leaf before it opens. It would seem therefore that the opening of this leaf coincides with some other change in the physiology of the seedling, resulting in the extrusion of the substance which stimulates infection by the nodule organism.

Summary and Abstract.

1. The appearance of nodules on seedlings of lucerne (*Medicago sativa*, L.) coincides with the opening of the first true leaf.

2. There is evidence that before this leaf opens the nodule bacteria do not as a rule infect the root hairs.

3. The delayed infection is due to the plant and not to any delay in the development of infective power by the bacteria.

4. When young inoculated seedlings, whose first leaves are still closed, are grown intermingled with older plants, a considerable number of nodules will develop on them, although scarcely any are formed on control seedlings of the same age, grown by themselves.

5. The solution surrounding the roots of seedlings whose first leaves are expanded has an influence in stimulating the appearance of nodules on younger seedlings, and increases the growth of the nodule organism on agar. The solution surrounding the roots of younger seedlings has no such effect.

6. The active substance inducing nodule appearance when the first leaf opens is not formed in this leaf, since the removal of the leaves while still closed has no effect on nodule appearance.

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The Vital Staining of Normal and Malignant Cells.—II. The Staining of Malignant Tumours with Trypan Blue.

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[PLATE 32.]

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1. *Introduction.*

In the attempt to discover a rational chemotherapeutic treatment for cancer, the influence of various dyes on malignant growths has been investigated. There has resulted a considerable difference of opinion as to the effect of *intra-vitam* staining upon tumour growth. Since many acid dyes when injected into the living animal readily stain certain types of cells, the present investigation was carried out primarily to determine whether malignant cells stain intra-vitally with trypan blue, as do their normal prototypes.

2. Intra-vitam Staining and Tumour Growth.

i. *Previous Work*.—The dyes which have been employed most in vital staining are isamin blue and trypan blue. According to von Möllendorff (1921) isamin blue is precipitated at the site of injection, and produces a localised vital coloration; it slowly passes into solution again and results in time in a general staining of the animal. Trypan blue, on the other hand, is one of the most suitable of the acid dyes for effecting direct intra-vitam staining.

W. Munck (1928) has recently published the result of an investigation on the influence of vital staining with trypan blue on the growth of transplanted carcinoma and sarcoma in the mouse. From his experiments he concludes that vital staining with trypan blue has a retarding influence on the growth of the tumours. The vitally stained animals did not live so long as unstained controls, but Munck considers this to be the result of the injections and not of the growth of the tumour.

In the papers of Bernhardt and Strauch (1928), Bernhardt (1928), and Roosen (1928), a similar retarding influence on tumour growth is attributed to isamin blue. Bernhardt, in his most recent paper, relates the result of injecting intravenously some 50 human cases of inoperable cancer with isamin blue. Some of these cases had previously received treatment, and had been radiated. The results of injecting the dye varied, but amongst the beneficial results he records an improvement of the general condition of the patient (appetite, vitality, reduction of cachexia), retrogression of the "Teilsymptomen" (*Ascites, Lymphstauungen und Drüsenpakete*), alleviation of pain, and marked regression of the tumour itself.

Other investigators have failed to obtain any definite therapeutic effects from the injection of dyes. Weil (1916) injected into tumour-bearing rats over 20 dyes of the benzidine group, including a series of synthetic compounds analogous to congo red. He found no specific retarding influence on the growth of the tumours. More recently Marsh and Simpson (1928) have investigated the influence on the growth of mouse tumours of 145 compounds, nearly all found in, or derivable from, coal-tar, including many vital dyes. They found no inhibition of tumour growth in non-lethal doses of any of the compounds they employed.

ii. *Personal Observations*.—My own experiments were directed primarily to determine the cytological results of injecting trypan blue into tumour-bearing mice. In view, however, of the conflicting results of previous workers it was considered desirable to investigate the effect of injecting trypan blue into

animals with moderately well-developed tumours (approximately 1 cm. in diameter). My procedure was to transplant tumours into a dozen mice, in some cases double this number; then when the tumours had attained an appreciable size, inject half of the animals with an aqueous solution of trypan blue. Experiments were carried out with three sarcomata and two carcinomata. The sarcomata were Nos. 37 and 2529 of this laboratory, and an experimentally-induced tumour originally obtained from Prof Fibiger. The carcinomata were No. 27 of this laboratory—a slow-growing adeno-carcinoma, and a hæmorrhagic carcinoma, 63. In none of the animals did the dye have any appreciable effect on the rate of growth of the tumours. Some showed a very slight retardation, but not sufficient to suggest that the dye had any specific action upon the tumour cells. It is to be remarked that Munck found only a very slight retardation of tumour growth when the dye was injected into animals with tumours.

When dye was injected into mice with tar carcinomata the tumours continued to grow, apparently at an unaltered rate, as indicated by their weekly charting. The same result was obtained with six mice bearing spontaneous tumours.

Trypan blue exerts a more marked influence on the growth of tumour grafts when injected soon after their transplantation. Munck injected mice with 0·5 per cent. trypan blue once a week, commencing within 2 days of transplanting tumours into them. The tumours grew considerably more slowly than the controls. I obtained similar results with the sarcoma, 37 S, and the adeno-carcinoma, 27, of this laboratory.

In addition to these experiments there is another way of approaching the problem, namely, to stain animals vitally and then to transplant the tumours. According to Lignac and Kreuzwendedich von dem Borne (1928), who have employed this method, the tumours then grow more quickly than the controls.

Munck also carried out similar experiments but with different results. He found no increase in the rate of growth when tumours were transplanted into vitally-coloured mice. Lignac and Kreuzwendedich von dem Borne maintain that the different result is due to differences in technique.

My own experiments were carried out with mice, which had received four injections of 0·5 c.c. of a 0·5 per cent. solution of trypan blue over a period of a fortnight. Thirty mice were vitally stained in this manner, but six of these died towards the end of the second week, so further injections were not made. The remaining 24 mice were divided into two groups. One dozen, together with 12 normal mice, were transplanted with the rapidly-growing sarcoma, 37 S.

The others, together with 12 normal mice, were transplanted with a slowly-growing adeno-carcinoma, 27, of this laboratory. The tumours in all cases were charted every week.

The results with the two tumours were different. With the rapidly-growing tumour, 37 S, at the end of the first week, the tumours of the vitally-stained animals were slightly smaller than those of the controls. This difference had vanished at the end of the second week. With the slower growing tumour 27, on the other hand, successful grafts take longer to become perceptible as growing tumours; consequently they cannot be charted until longer than a week after transplanting. From the time of the first charting of these tumours, and throughout the period of their growth, no differences were found in their rates of growth in the vitally-stained and the control animals.

It seems highly probable that the explanation of these results is to be found in the action which the trypan blue has upon the tumour stroma rather than upon the malignant cells. This point will be discussed later, after dealing with the cytological results of this investigation.

3. *The Cytology of Vitally-stained Tumours.*

i. *Previous Work.*—Most of the previous research work has been confined to the influence of vital staining upon the rate of growth of tumours. Little attention has been paid to the cytological effect of the dye upon the malignant cells. In his study of vital staining with carmine, Kiyono (1914) found that tumour cells of a primary fowl sarcoma showed the same granular dye deposits as fibroblasts. This property of the sarcoma cells was retained after transplantation. Epithelial tumour cells of the mouse and cells of a fowl carcinoma, however, did not stain intra-vitally with carmine. Weil (1916) working with dyes of the benzidine group—congo red and analogous compounds—concluded that “living tumour cells are not penetrated by colloidal dyes,” although necrotic areas of tumours are intensely coloured in vital staining.

Karczag, Teschler and Barok (1924) employing acid dyestuffs of the triphenylmethane group—Fuchsin, S, Lichtgrün and Wasserblau—did not obtain any vital colouring of tumour cells; a result with which the recent work of Györgyi (1928) is in agreement. Engel (1925) has disputed this result. He maintains that transplantable sarcoma cells can be vitally stained with these dyes, but not carcinoma cells. In most of his experiments 1 c.c. of a 1 per cent. solution of the dyestuff was injected subcutaneously into mice, approximately 15, 5 and 1 hour before the animal was killed, and examined histologically. He found a diffuse staining of the tumour cells and not the typical granular

staining of vitally-stained cells. Bernhardt and Strauch (1928), who injected isamin blue into human cancerous subjects, did not find any staining of the cancer cells. The dye was taken up by certain cells, which they consider correspond to the "pyrrol cells of Goldmann."

ii. *Personal Observations.* (a) *Technique.*—In studying the cytological action of trypan blue on tumour-bearing mice, aqueous solutions of the dye have been injected subcutaneously, intraperitoneally, and also into the tumours. The smallest injection employed has been 0.5 c.c. of a 0.5 per cent. solution of trypan blue in distilled water. The largest dosage has been six injections of a 1 per cent. solution over a period of a fortnight. The smallest amount of dye injected into a tumour has been a little less than 0.5 c.c. of a 0.5 per cent. solution, and the largest amount three injections of 0.5 c.c. of a 2 per cent. solution during the course of a week. Animals have been killed and tissues fixed and examined at various times after the last injection of the dyestuff. Examinations of the tissues have been made 1, 6, 18 and 24 hours after the last injection. The method of injection and the cytological technique employed have been fully described in my former paper (Ludford, 1928). Besides the tumours the liver and kidney of each animal have been examined.

During the course of this work nearly 60 tumour-bearing mice have been examined histologically after varying dosages of dyestuff. Detailed tables have been drawn up of the extent of the vital staining of the tumours, dermis, kidney and liver. No useful purpose would be served by publishing these results in detail, so that in the following description reference will be made only to those cases which are of special cytological interest.

(b) *General Results.*—Only in rare cases have the actively-growing transplanted tumour cells been found to stain *intra-vitam* with trypan blue. The necrotic areas of tumours are usually well stained, especially with large injections of the dye.

The intensity of the staining of the normal tissue is largely determined by the amount of dye that has been injected. Spontaneous adeno-carcinomata and tar cancers of mice frequently show isolated cells, and groups of cells, with the typical granular staining. The number of stained cells varies with different tumours. In those which I have examined the great majority of the parenchyma cells have been unstained.

4. *The Morphology of the Staining Process.*

i. *Epidermal Cells and Carcinoma Cells.*—There are very few references in the literature to the stainability of epidermal cells by acid dyes. Von Möllen-

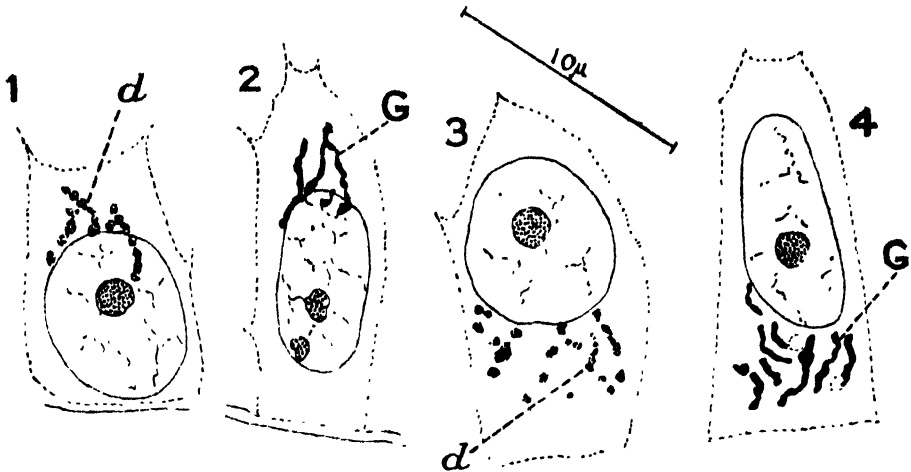
dorff (1921) states that the only epidermal cells that stain with acid dyes are those of the mammary glands. Glassunow (1928), who studied the cytology of vital staining with trypan blue in the guinea-pig, came to the same conclusion. In young mice, however, Blotevogel (1924¹) found a slight granular staining of the epithelial cells of the cornea with trypan blue. The same worker (1924²) also studied tooth-development in the mouse by the aid of vital-staining technique. He found trypan blue accumulated in the ectodermal cells of the enamel organ. The dye droplets occurred in the cytoplasm above the nucleus. Blotevogel regards this staining as an expression of the high degree of metabolic activity of the cells.

I have already pointed out that the literature contains no description of the vital staining of carcinoma cells by acid dyes. In my experiments a slight staining of epidermal cells has been observed in mice bearing tumours, which have received relatively large injections of trypan blue into the tumours. Following two injections of 0.5 c.c. of a 2 per cent. solution of the dye into the tumour pale blue granulations are visible in the overlying epidermis. The dye droplets (*d*) are usually grouped towards the upper pole of the nucleus, as shown in the text-fig. 1. Their grouping is often strikingly similar to the form of the Golgi apparatus (*G*), as is seen by comparing figs. 1 and 2.

With injections of 2 per cent. dye solution, when the tumour is near the surface, groups of coloured droplets frequently occur at the opposite pole of the nucleus. Preparations in which the Golgi apparatus has been impregnated show that it also, in many cases, is reversed in polarity. This is illustrated in figs. 3 and 4. Such coincidence between the position and form of the Golgi apparatus and the distribution of acid dye droplets lends support to the view expressed in my former paper that the Golgi apparatus brings about the segregation of the dyestuff.

Examination of sections of actively-growing carcinomata has failed to reveal any marked staining of the healthy tumour cells. Cells of the hæmorrhagic carcinoma, 63, failed to stain. A large number of experiments have been carried out with a transplantable adeno-carcinoma (27); but only occasionally has the milky secretion in healthy tumour cells stained faintly blue. On the other hand, broken-down cellular *débris*, containing products of secretion of the cells (milk-like secretion), is usually well stained.

The cells of tar tumours of the mouse, in general, fail to stain with trypan blue. Occasionally in tar carcinomata one finds stained cells. It is often difficult to be quite sure whether these are carcinoma cells, or cells of the stroma. In rare cases dye droplets are discernible in dividing cells, and during



TEXT-FIGS. 1-4.—Epidermal cells stained *intra-vitum* with trypan blue, showing the relationship between the arrangement of the dye droplets (*d*) and the form of the Golgi apparatus (*G*). These cells are drawn from section of the skin overlying a large transplantable tumour (37 S) into which has been injected a 2 per cent. dye solution.

Protocols of Experiments.—Figs. 1 and 2, a little less than half a cubic centimetre, of 2 per cent. dye solution injected into the tumour on the first and second days of the experiment; mouse killed and examined 48 hours after the last injection. Figs. 3 and 4, the same amounts of dye injected into the tumour on the first and second days; repeated on the seventh day; on the 14th day 0.5 c.c. of 0.5 per cent. solution of dye injected subcutaneously; animal killed on the 15th day. Figs. 1 and 3 from frozen sections. Figs. 2 and 4 drawn from modified Kopsch preparations.

keratinisation with the breaking-down of cells some staining occurs. The cells which are stained, however, constitute an almost negligible minority compared with those which do not stain at all. Within the stained cells the dye almost always occurs in the form of granules, grouped towards one pole of the nucleus as in the normal cells.

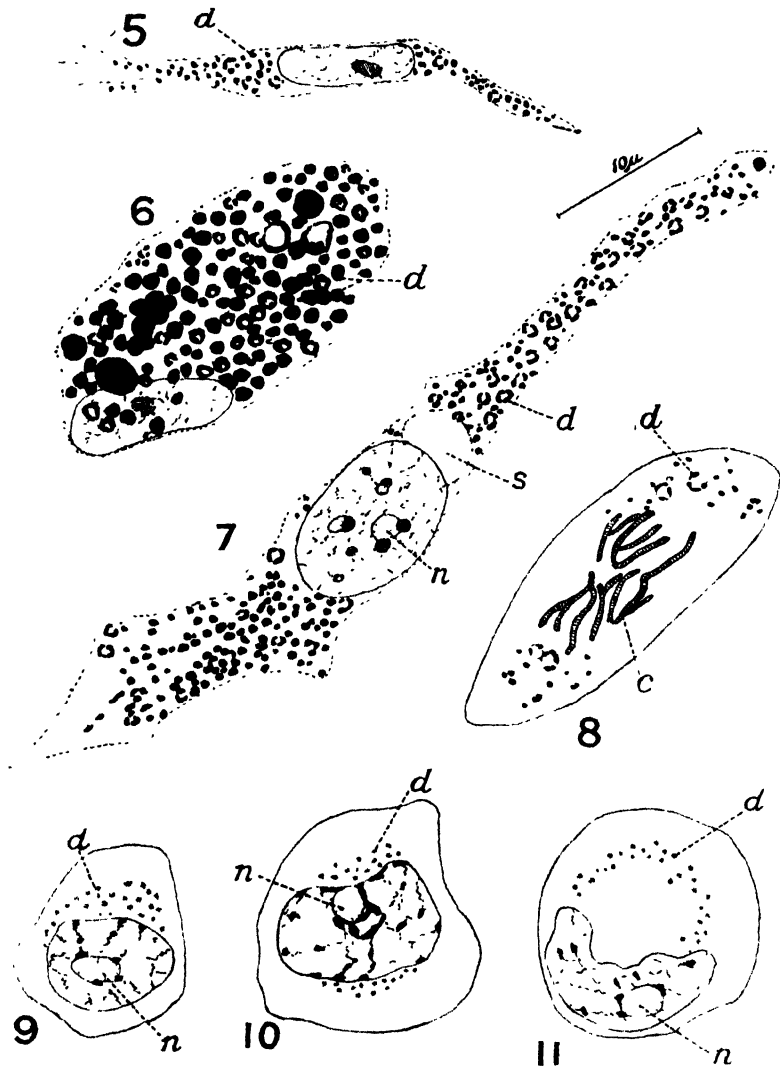
In spontaneous adeno-carcinomata (*mammary tumours*) relatively large areas of the tumour may show stained cells. When such cells are arranged in acini the dye droplets are as a rule grouped between the nucleus and the lumen of the acinus. Otherwise the dye occurs in the form of a single droplet, or group of smaller droplets at one pole of the nucleus, an arrangement which coincides in position with that of the Golgi apparatus. This mode of staining resembles the staining of milk in the glandular cells of the lactating mammary gland, and indicates that the segregation of the dye, which has penetrated the cytoplasm, together with the milky-secretion, occurs in this part of the cytoplasm.

ii. *Fibroblasts and Sarcoma Cells*.—The classical researches of Goldmann constitute the basis of our knowledge of the vital staining of the connective tissues. More recently Evans and Scott (1921) have investigated cytologically the behaviour of connective tissue cells towards a large number of acid dyes of the azo class. They found a marked difference between the staining of fibroblasts and macrophages. They say "the power to store vital dyestuffs is, on the part of the macrophage, greatly in excess of a similar capacity shown by the fibroblast cell." The difference is seen in "the size, form and number of vital dye 'granules.'"

Connective tissue cells of the mouse stain readily with dilute aqueous solutions of trypan blue. If one gives a mouse, bearing a transplantable sarcoma, four injections of 0·5 c.c. of a 0·5 per cent. solution of trypan blue over a period of a fortnight, the macrophages and fibroblasts surrounding the tumour, and also those of the stroma, are found deeply stained, while almost all the malignant cells remain uncoloured. Only the necrotic tumour cells take up the dye to any extent.

The same results follow the injection of stronger solutions of dye into the tumour. Text-figs. 5, 6, 7 and 9, 10 and 11 are all drawn from a mouse bearing the transplantable sarcoma 37 of this laboratory. The mouse received a little less than 0·5 c.c. of a 2 per cent. trypan blue solution into the tumour on the first and second days of the experiment. Two days later it was killed and examined histologically. The centre of the tumour was necrotic and stained blue; this central region was surrounded by an area of injured cells, most of which were unstained. The nuclei of the cells showed peripheral clumping of the chromatin. A few late abnormal mitotic figures were seen—apparently the effect of the dye on cells actually dividing, or about to divide, at the time of the injection. External to these injured cells were actively-growing sarcoma cells, amongst which numerous normal mitoses could be seen. The tumour cells which stained occurred principally at the margin of the area of injury. Three of these cells are represented in the figs. 9, 10 and 11. They have pale blue dye droplets (*d*) grouped around the sphere, which, in sections stained with neutral red, appears paler than the rest of the cytoplasm.

Comparison with Golgi apparatus preparations shows that the dyestuff is segregated in that part of the cytoplasm where the apparatus is situated. The nucleolus (*n*) of these cells also has a pale blue tint. The tumour is surrounded by a region of intensely stained cells, specially marked on the epidermal side. The appearance of the normal fibroblast is indicated in fig. 5 (*d*-dye droplet). An hypertrophied fibroblast is shown in fig. 7. It has several



TEXT-FIGS. 5-11.—Fibroblasts, macrophages and sarcoma cells stained *intra-vitam* with trypan blue. Fig. 5, normal fibroblast; fig. 7, hypertrophied fibroblast; fig. 8, fibroblast undergoing mitosis; fig. 6, macrophage distended with dye droplets (*d*); figs. 9, 10, 11, faintly stained sarcoma cells from the margin of a necrotic area of sarcoma 37.

Protocols of Experiments.—Fig. 8, the same as figs. 12 to 16. Other figures, the same as figs. 1 and 2. *c*, chromosome; *d*, dye droplet; *n*, pale blue nucleolus; *s*, sphere. (All figures drawn from frozen sections.)

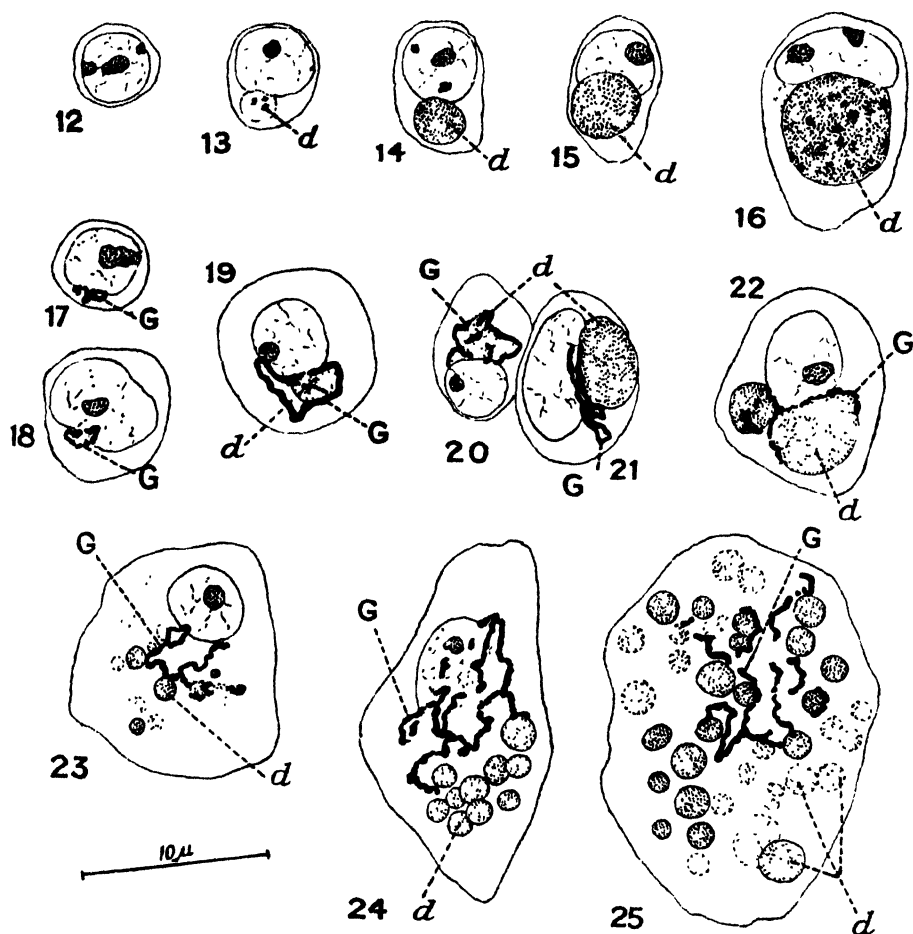
nucleoli (*n*) stained faintly bluish. Slightly stained fibroblasts are capable of division. One actually dividing is shown in fig. 8. A typical histiocyte is

seen in fig. 6. It will be noticed that it has much larger accumulations of the dyestuff (*d*) than the fibroblasts. Within the tumour are a considerable number of macrophages; and their arrangement seems to suggest that they may have migrated into the tumour from the dermis. Worthy of note is the fact that the mouse at the time of killing was in a dying condition. Examination of its liver and kidney showed large areas of necrosis. In the kidney most of the convoluted tubules had been destroyed by the large accumulations within them of the dyestuff.

iii. *Cells of the Stroma and Tissues Surrounding Tumours*.—The staining of the fibroblasts and macrophages, to which reference has just been made, is seen in all tumours, both spontaneous and transplantable. Goldmann first drew attention to the accumulation of macrophages, called by him “Pyrrhollen,” in places where tumour implantation had taken place. But fibroblasts and macrophages are not the only cells in the tissues surrounding tumours which stain *intra-vitam* with trypan blue. As in general inflammatory conditions “large quantities of lymphocytes and monocytes migrate out of the blood vessels.” “In the later stages the monocytes and lymphocytes both transform themselves into larger cells, the polyblasts.” Ultimately “the hypertrophy of the hæmatogenous cells reaches such a degree that they can no longer be separated from the mobilised local histiocytes: simultaneously they show a rapidly increasing accumulation of the vital dye (Tschaschin (1913), Downey (1917)).”

I have quoted here from Maximow’s description of the behaviour of lymphocytes in inflammatory reactions in his recent review of “Lymphocytes and Plasma cells” (1928). The same process occurs to a varying extent in the tissues surrounding tumours, and is mainly responsible for the intense blue coloration around tumours of many vitally stained animals.

Various types of cells from the reaction tissues around tumours are shown in text-figs. 12 to 25. Figs. 12 to 16 were drawn from frozen sections of a tar tumour of a mouse which had received six injections of 0.5 c.c. of a 0.5 per cent. solution of trypan blue over a period of 17 days. Fig. 12 represents a typical lymphocyte from a blood capillary. Fig. 13 shows a slightly larger cell, containing a single vacuole, inside which are three pale blue droplets (*d*). Figs. 14, 15 and 16 show the progressive deposition of dyestuff resulting in the formation of a single large dye inclusion. The remaining figs. 17 to 25 are all drawn from sections of a transplantable sarcoma (37 S) of a mouse which had had two injections, of a little less than 0.5 c.c. of a 2 per cent. solution of the dye, injected into the tumour three times, and 0.5 c.c. of a 0.5 per cent.



TEXT-FIGS. 12-25. — Transformed blood cells (lymphocytes and monocytes), and macrophages stained *intra-vitam* with trypan blue. Figs. 12-16 are drawn from sections of a tar tumour. Figs. 17-25 from sections of a transplantable sarcoma (37). Fig. 12, lymphocyte from a blood capillary. Fig. 13-16, extravasated cells of lymphocytic origin showing the increasing accumulation of dye (*d*) with enlargement of the cell. Figs. 17 and 18, lymphocyte and monocyte showing the Golgi apparatus (*G*). Figs. 19-22, similar series of cells as figs. 12-16, showing the relationship between the first-formed dye droplets (*d*) and the Golgi apparatus (*G*). Figs. 23, 24 and 25, macrophages showing the Golgi apparatus (*G*) and dye droplets (*d*).

Protocols of Experiments.—Figs. 12-16, 0.5 c.c. of 0.5 per cent. solution of trypan blue injected subcutaneously 1st, 4th, 7th, 10th, 14th, 17th days from beginning of experiment; mouse killed on the 18th day. Figs. 17-25, the same as for figs. 3 and 4. (Figs. 12-16 drawn from frozen sections; figs. 17 to 25 from modified Kopsch preparations.)

solution subcutaneously 24 hours before the animal was killed. (Intra-tumoural injections, 1st, 2nd and 7th days ; subcutaneous injection, 14th day ; killed, 15th day from beginning of experiment.)

The figs. 17 to 25 are drawn from sections prepared by the modified Kopsch method described in my former paper (Ludford, 1928). This method as I have pointed out enables one in successful preparations to demonstrate both the Golgi apparatus and dye inclusions. Fig. 17 represents a lymphocyte from a blood capillary showing the small Golgi apparatus (G). Fig. 18 shows a monocyte with a similar type of Golgi apparatus (G). The remaining figs. 19 to 25 show various transition stages between cells of the lymphocyte type and macrophages.

Figs. 19 and 20 represent the first stages in the segregation of the dye by the cells. The Golgi apparatus (G) of these cells is enlarged and surrounds light-blue dye droplets (*d*). Figs. 21 and 22 show deep-blue dye droplets (*d*) formed in relationship with the Golgi apparatus (G). Comparison of figs. 12 to 16 with figs. 17 to 22 shows that the segregation of the dye by these cells takes place in the same manner in widely different tumours, and that the dye deposits in the cells present the same appearance in tissues fixed in formol and cut frozen as in preparations made by the modified Kopsch technique.

The occurrence of large numbers of cells of the types illustrated in figs. 12 to 22 suggests that the first formed dye-droplets are segregated from the cytoplasm in relationship with the Golgi apparatus. The same mechanism of segregation appears to be functioning in the larger cells, shown in figs. 23 and 24. Macrophages, however, can phagocytose relatively large particles besides segregating the diffuse dyestuff into droplets. Fig. 25 represents a macrophage in which dye droplets are being formed in association with the Golgi apparatus (G), but in addition large droplets (*d*) are present throughout the cytoplasm. After a certain amount of dyestuff has been taken up by a macrophage the segregation action of the Golgi apparatus breaks down, dye then accumulates throughout the cytoplasm. Evans and Scott (1921) have shown that this accumulation may take place to such an extent that the dyestuff may crystallise out in the cytoplasm.

The taking-up of trypan blue by local fibroblasts and histiocytes, and by transformed blood cells, results in the deeply-stained areas around many tumours. There is considerable variation in the extent of this staining, as can be seen by reference to the photomicrographs of Plate 32. The margin of a tar tumour is there shown in fig. 1. On the left and along the bottom of this figure is seen the tumour. The rounded blackened cells are mostly macrophages, which have

been stained by the trypan blue. The mouse bearing this tumour had received six injections of 0·5 c.c. of a 0·5 per cent. solution of dyestuff over a period of 18 days. The cells represented in text-figs. 12 to 16 were drawn from sections of this same tumour.

Fig. 2 of Plate 32 shows a part of the margin of a large spontaneous carcinoma of the mouse, which had received four injections of 1 c.c. of 0·5 per cent. solution of trypan blue over a period of 10 days, and the photomicrograph represents the condition 4 hours after the last injection. The tumour is seen at the upper left and lower right-hand corners of the photograph. Extending diagonally from the lower left-hand corner are seen large numbers of deeply-stained cells, mostly macrophages with fewer fibroblasts.

The edge of a metastasis of the same tumour in the lung is shown in fig. 3. From the lower left-hand corner there runs diagonally a bronchiole which is surrounded by fibroblasts which are stained. On either side are tumour cells mostly unstained. The metastasis is surrounded by a fine capsule of connective tissue, the fibroblasts of which are well stained. Such secondary growths in the lungs therefore appear as blue nodules when the thorax is opened *post-mortem*. Comparison of fig. 2 with fig. 3 (Plate 32) shows a remarkable difference in the occurrence of numerous macrophages around and within the tumour, and their complete absence from the metastasis.

Three other cases of lung metastases have occurred in animals which I have stained vitally. They have been exactly the same in this respect. Fig. 4 of Plate 32 shows the margin of a secondary growth of a tar carcinoma which has destroyed the right axillary lymph node. The mouse from which this tumour was taken had, previous to death, received two subcutaneous injections of 1 c.c. of a 0·5 per cent. solution of trypan blue on two alternate days. Its general condition was such as to prevent further injections being made. The photomicrograph shows on the left deeply-stained macrophages and fibroblasts, to the right of which are unstained tumour cells, amongst them being a few macrophages. A zone of necrotic cells stained by the dye is seen on the extreme right of the figure.

The variation in the amount of deeply-stained tissues around different types of tumours is not without significance in the chemotherapy of cancer. This aspect of the subject will be discussed in a following section of this paper. Relevant in this connection are the following conclusions :—

- (i) Spontaneous tumours (including tar tumours) are surrounded by a much larger zone of vitally-stained cells than are most transplantable tumours.

- (ii) Very slow-growing transplantable tumours resemble, in this respect, spontaneous tumours, rather than the more rapidly-growing transplantable ones.
- (iii) The primary tumour and its metastases may differ considerably in the extent of the local macrophage reaction they induce. The difference between primary tumours and lung metastases is specially striking.

5. *Physico-chemical Aspects.*

There are two factors involved in vital staining with acid dyes :--

- (i) The ability of the dye to penetrate into the cell.
- (ii) The segregation of the dyestuff by the cytoplasm in a non-diffusible form.

It is impossible to tell whether the dye penetrates the malignant cell in small quantities, because it would not be detectable microscopically. There is, of course, the possibility that the dyestuff may be destroyed by the cell. To investigate this point fine suspensions were prepared of tumour, kidney, and liver, by crushing these tissues in a mortar with sterile sand and normal saline. Small quantities of each suspension were pipetted off into a separate test-tube, containing a very dilute solution of trypan blue in normal saline. The three test-tubes were then incubated at body temperature for 24 hours. Examination of the tubes at various intervals failed to show any appreciable difference in the three tubes. It therefore seems clear that the failure of tumour cells to stain vitally with trypan blue is not due to the cells destroying the dyestuff.

There remains the probability that tumour cells do not segregate, in a non-diffusible form, the dye which has penetrated their cytoplasm. In my former paper (Ludford (1928)), and also in preceding paragraphs of this communication, I have brought forward evidence in support of the hypothesis that the Golgi apparatus is specially concerned in the process of segregation and elimination. Now it is particularly noticeable in working with tumours cytologically that the Golgi apparatus in them is easier to impregnate than in the non-malignant cellular prototypes of the tumour. It has been my experience that almost all tumour cells have a well-developed Golgi apparatus. One cannot, therefore, exclude altogether the idea that malignant cells may be better able to segregate and eliminate the dyestuff, and hence prevent its accumulation within their cytoplasm.

There is another aspect of the process of segregation which most likely explains the non-staining of actively-growing tumour cells. Trypan blue is a semi-colloidal dyestuff. Flocculation of a colloid occurs by neutralisation of

the electrical charge on its micellæ. The micellæ of trypan blue bear a negative charge; they will therefore be discharged on being brought in contact with positively charged particles. That is to say, trypan blue will be flocculated or precipitated in the presence of a more alkaline medium. According to Lewis (1928) tumour cells have a lower pH than normal cells (freshly excised tumours pH — 6·8), probably due to their glycolytic activities resulting in the production of lactic acid. The failure of tumour cells to stain vitally with trypan blue may thus be the result of the more acid cytoplasm of such cells, and be another expression of their metabolism, to which Warburg has drawn attention.

6. The Bearing of Vital Staining with Acid Dyes on the Chemotherapy of Cancer.

Intra-vitam staining with trypan blue enables us to trace the distribution of a semi-colloid introduced into the animal body. Above all it picks out the macrophage system—the histiocytes of the connective tissues, the stem cells of the liver, the reticular cells of lymph nodes, and bone marrow, and also the endothelial cells of the lymph sinuses and bone marrow. Other types of cells, such as those of the liver and kidney, stain as well. The dye stuff accumulates in the cytoplasm of living cells in the form of granules, but dead cells stain diffusely. The nucleus does not stain during life.

When a tumour-bearing animal is injected with trypan blue, we find the tissue around the tumour and the stroma becomes stained, but actively-growing tumour cells remain uncoloured. Only in certain spontaneous tumours do we find groups of cells with typical granular staining. The absence of uniformity in the staining of spontaneous tumours is undoubtedly an expression of the wider variation in the state of the cells in different areas of the tumours compared with transplantable tumours.

When relatively strong concentrations (2 per cent.) of dyestuff are injected into tumours, central necrosis occurs; but a peripheral layer of unstained tumour cells continues to grow. Mice which have received such injections do not live long, as strong concentrations of trypan blue are toxic. The dye is excreted in the urine and bile, as I described in my former paper (Ludford, (1928)). Thus it comes about that, when excess of dyestuff collects in the liver and kidney cells, in the course of its elimination the cells are killed: consequently we find almost complete destruction of the convoluted tubules of the kidney and wide areas of necrosis in the liver parenchyma. There is a simultaneous destruction of macrophages in the tissues surrounding the tumour.

According to Schulemann (1917) most metallic colloids, and suspensoid

colloid such as India ink, and fine suspensions of cinnobar, behave in the same way as acid dyestuffs when injected into animals. The only property in common possessed by these media is the negative electrical charge borne by the particles (colloidal micellæ). We should expect, therefore, that metallic colloids injected into a tumour-bearing animal would become distributed in the same way as acid dyes. Various metallic colloids have been employed in the treatment of cancer, and also acid dyes, as described in this paper. Searle (1920), in a review of the use of colloids in medicine, mentions that colloidal copper and iodine have been employed intra-venously, and colloidal sulphur subcutaneously in the treatment of cancer; also much discussed recently is the colloidal lead treatment, introduced by Blair Bell.

On the basis of the results of vital staining with acid dyes, one would expect that any therapeutic action such colloids might exert would not primarily be upon the malignant cells, but upon the cells of the stroma and surrounding tissues, especially cells of the macrophage type. This also seems to be the explanation of the retarding influence trypan blue exerts upon transplantable tumours, when injections of this dye are commenced immediately after transplantation.

Reference has already been made in this communication to the variation in the amount of stained tissues in and around different types of tumours (see figs. 1, 2, 3 and 4, Plate 32). It is to be expected that injections of metallic colloids would result in corresponding differences in their distribution, and hence in their possible influence upon the growth of various tumours. The difference between the vital staining of spontaneous tumours and lung metastases (*cf.* figs. 2 and 4) is particularly striking and has important clinical implications.

7. *Summary.*

1. Vital staining of tumour-bearing mice with trypan blue has no appreciable influence on the rate of growth of well-organised transplantable or spontaneous tumours.

2. Injections of trypan blue, sufficient to stain intensely the fibroblasts and histiocytes (macrophages) of the connective tissues (text-figs. 5 to 8), fail to stain actively-growing sarcoma cells. (Tumours 37 and 2529 of the Imperial Cancer Research Fund, and Fibiger's tar sarcoma.)

3. Necrotic areas of transplantable sarcomata stain with varying degrees of intensity. Tumour cells around the margin of such areas occasionally show faintly-stained granules grouped round the sphere (figs. 9, 10, 11).

4. It is exceptional for epidermal cells to be stained vitally by acid dyes.

Some staining of epidermal cells has occurred when a relatively strong solution of trypan blue (2 per cent.) has been injected two or three times into tumours growing close up to the epidermis (figs. 1 to 4).

5. The results of vitally staining mice bearing carcinomata of various types are as follows :—

- (a) Transplantable hæmorrhagic carcinoma, 63—no staining of tumour cells.
- (b) Transplantable adeno-carcinoma, 27 (mammary gland tumour)—occasional faint staining of milky secretion in healthy tumour cells, but the staining is very slight in comparison with the staining of the milk in the lactating mammary gland.
- (c) Spontaneous mammary adeno-carcinomata—relatively large areas of these tumours may contain stained droplets, suggesting the staining of a milky secretion. Here again the staining is much less intense than in the mammary gland.
- (d) Tar cancer—occasionally one finds stained cells, but this is relatively rare. During keratinization with the breaking-down of cells some staining often occurs. The absence of uniformity in the staining of spontaneous and tar tumours is attributed to the wider variation in the state of the cells in different areas of these tumours as compared with transplantable tumours. In all types of tumours necrotic areas are stained.

6. It is possible that most malignant cells fail to stain like their normal prototypes, because their greater acidity prevents the flocculation of acid dyes. Impermeability of the cell membrane, or a more rapid method of segregating and eliminating any dyestuff which entered the cells, would also account for their non-stainability.

7. Deeply-stained areas occur around many tumours, due to the taking-up of the dyestuff by local fibroblasts and histiocytes, and by transformed blood cells (lymphocytes and monocytes). The stroma of tumours also is stained. The manner in which transformed blood cells are stained is shown in figs. 12 to 22. Cells of the macrophage type segregate the dyestuff, which penetrates their cytoplasm in solution, and also phagocytose the dye in particulate form (figs. 23 to 25). The intensity of the macrophage reaction around different types of tumours varies considerably. It has been found that :—

- (i) Spontaneous tumours (including tar tumours) are surrounded by a much larger zone of vitally-stained cells than are most transplantable tumours (Plate 32, figs. 1 and 2).

- (ii) Very slow-growing transplantable tumours resemble, in this respect, spontaneous tumours rather than the more rapidly-growing transplantable ones.
- (iii) A primary tumour and its metastases often differ considerably in the extent of the local macrophage reaction they induce. The difference between primary tumours and lung metastases is specially striking (*cf.* figs. 2 and 3, Plate 32).

Since most metal colloids when introduced into the animal body become distributed throughout the tissues in the same way as acid dyes, it is suggested that these differences may be of considerable significance in the chemotherapy of cancer.

8. When two or three injections of a relatively concentrated solution of trypan blue (2 per cent.) are made into a transplanted tumour, considerable necrosis of the tumour cells results. A peripheral zone of tumour cells, however, continues to grow. Mice so treated do not live long, since the dye which would be excreted by the liver and kidneys collects in these organs to such an extent as to cause considerable cellular destruction.

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DESCRIPTION OF PLATE 32.

Photomicrographs of the margins of vitally-stained tumours, showing the variation in the number of stained cells around different types of growths. The original preparations are frozen sections counterstained with neutral red. The photomicrographs were taken at a magnification of 140, using a red screen to intensify the blue staining and reduce the red. The *intra-vitam* stained cells therefore appear black.

I am indebted to Mr. F. J. Pittock, of the Department of Embryology, University College, for these photographs.

FIG. 1.—Margin of a tar tumour of the mouse. Protocol of experiment—0·5 c.c. of a 0·5 per cent. solution of trypan blue injected subcutaneously on the 1st, 4th, 7th, 10th, 14th and 17th days from the beginning of the experiment ; the animal killed on the 18th day.

FIG. 2.—Margin of a spontaneous adeno-carcinoma (mammary tumour). Protocol of experiment—Injected subcutaneously—1 c.c. of a 0·5 per cent. solution of trypan blue on the 1st, 3rd, 6th and 10th days from the beginning of the experiment ; mouse killed 4 hours after the last injection.

FIG. 3.—Lung metastasis of the same tumour as fig. 2.

FIG. 4.—Metastasis of a tar tumour which has almost destroyed the right axillary lymph node. Protocol of experiment—1 c.c. of a 0·5 per cent. solution of trypan blue on the 1st and 3rd days ; mouse killed on the 4th day.

The Influence of Witte's "Peptone" and of Digestion of Blood Platelets and Plasma.

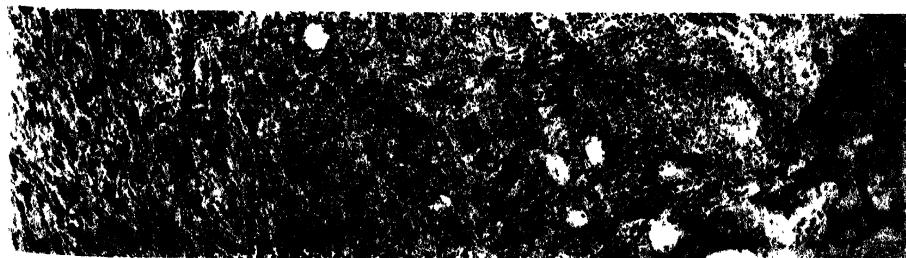
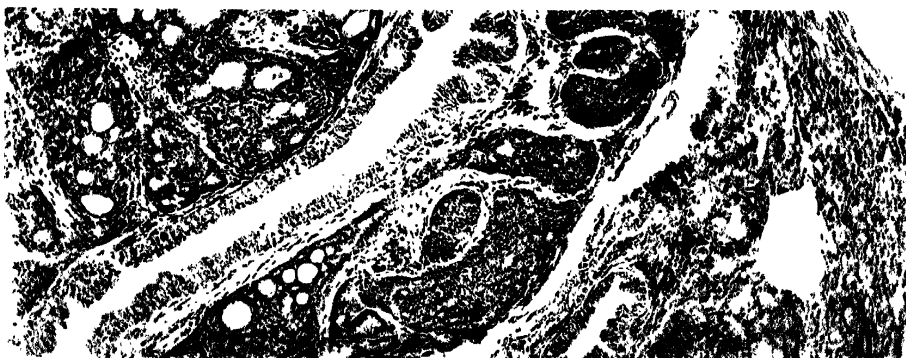
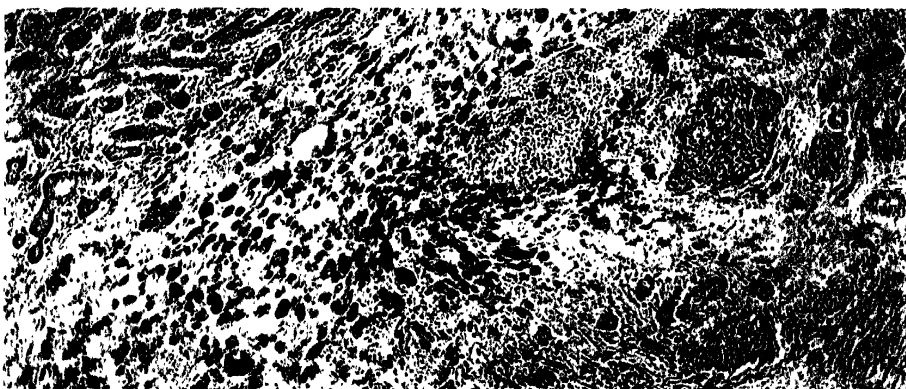
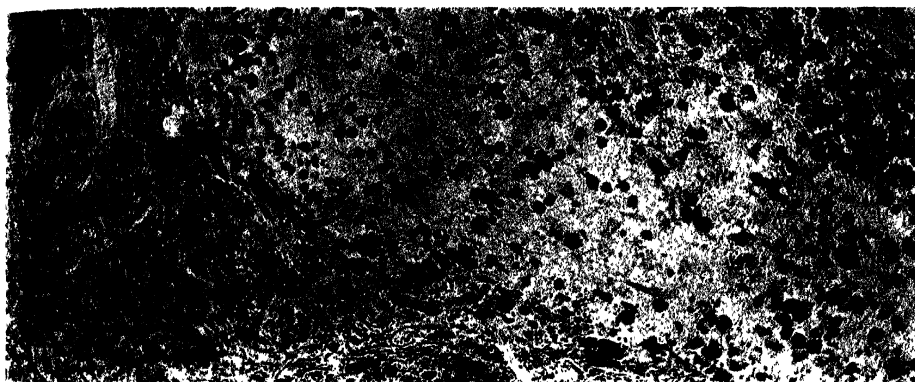
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Summary of Recent Work.

Experiments have been described to the Society which show that the anticoagulant action of Witte's "peptone" may be as great on shed blood as on circulating blood, if the blood is drawn through a paraffined cannula into a paraffined vessel. It was also recorded that a moderate concentration of "peptone" (0·3 per cent.) does not suppress the clotting of blood which has remained in contact with glass for 2 or 3 minutes, although this amount of the anticoagulant suffices for the prolonged inhibition of clotting when either injected intravenously or added to blood in paraffined vessels. From these observations it was concluded that the anticoagulant action of "peptone" can be exerted without stimulating a secretion of antithrombin, either in the



liver or vascular endothelium. It was also suggested that "peptone" reacts with plasma and temporarily stabilises that fluid (Pickering and Hewitt, 1, 2).

In a recent review of this work, Nolf (3) admits the coagulant action of "peptone" on shed blood, but maintains that it is fundamentally different from that produced when "peptone" is injected into the blood stream. Several facts are inconsistent with this conclusion. Blood withdrawn after the intravascular injection of "peptone" exhibits the following characteristics: It is coagulated by the passage of a stream of carbon dioxide, by acidification, by dilution with distilled water, by the addition of chemically inert powders and by filtration through a clay cell (Fano (4), Wooldridge (5), Gratia (6)). All these characteristics are exhibited by bloods or plasmas which have been kept fluid by the addition of small amounts of "peptone" after shedding (Pickering (7)).

In a paper published last year, Barratt (8) confirms the anticoagulant action of "peptone" *in vitro*. He states that such action is exerted on fibrinogen and thus differs from that of antithrombins which prevent blood clotting by neutralising thrombin. The fibrinogen used in these experiments was not, however, completely separated from prothrombin. All that can, at present, be said of this work is that it indicates that the anticoagulant action of "peptone" is exercised on the fraction of plasma containing fibrinogen and prothrombin, in a manner different from the action of antithrombin.

A different conclusion is reached by Mills (9), although he states that he has uniformly confirmed the observations of Pickering and Hewitt on the anticoagulant action of Witte's "peptone" on freshly drawn blood. Mills finds that "peptone" slightly retards the clotting of citrated and "re-calcified" plasma, but he states that "peptone" is without such action when the platelets have been removed. He concludes that the anticoagulant action of "peptone" on freshly drawn blood is solely due to the suppression of the lysis of platelets and he rejects the conclusion that the plasma participates in the stabilisation of blood by peptone.

The experiments recorded in this communication were devised to test the value of these suggestions.

Technique.

Early in these experiments it was noted that the clumping and lysis of platelets are not only hastened by contact of the blood with surfaces which it wets but also by exposure of the plasma, for 50 seconds or longer, to the air. All operations, except the fixing and staining of platelets, were therefore con-

ducted without exposing the plasma or platelets to either surfaces wetted by blood or to the air.

The writer's own blood was used. It was obtained by venepuncture, with paraffined syringes, and was immediately transferred into paraffined centrifuge tubes, the blood being passed into the tubes under liquid paraffin. Centrifuging, under liquid paraffin, gave a suspension of platelets in a plasma free from other corpuscles, and prolonged centrifuging of this plasma yielded a deposit of platelets.

The transference of plasma or of platelets from one medium to another was effected by passage through pipettes lined with solid paraffin, which were filled with liquid paraffin. Suction and ejection was accomplished without expelling all the liquid paraffin from the pipettes. A cushion of fluid paraffin thus afforded protection against exposure to the air. The contents of the pipettes were discharged under liquid paraffin into paraffined vessels.

When Witte's "peptone" was added to the blood, sufficient of the anti-coagulant, dissolved in 0.875 per cent. NaCl, was placed in the syringe to provide a concentration of 0.3 per cent. and was mixed with the blood as it passed into the syringe.

The rapidity of the clumping and lysis of platelets was estimated by serial microscopic examinations, at intervals of 1 minute, of both unstained and stained corpuscles. The latter were fixed and stained with formalin and brilliant cresyl blue, in accordance with the technique of Rees and Ecker (10).

Observations were made with plasma and platelets obtained at the same time each day, 14 hours after fasting, $3\frac{1}{2}$ hours after the consumption of 300 grams of roasted lean beef, and similarly after the ingestion of 300 grams of polished rice, which had been boiled after the addition of a little sugar.

Experiments.

The results obtained are summarised in the following notes and tables.

The rapid clumping of platelets is invariably followed by their rapid lysis. The converse is also true. In Table I the minimum times recorded for clumping should be read with the minimum times noted for the lysis of platelets and for the gelation of plasma. The maximum times also correspond.

Control experiments showed that the passage of a suspension of platelets through a paraffined pipette containing air decreases resistance to clumping and lysis, reduces the response in platelets to the stabilisation of blood by "peptone," and partially masks the differences between the agglutinability and stability of platelets during fasting and the digestion of a meat meal.

Table I.—Illustrating the Maximum and Minimum Times (in minutes) required for the clumping and lysis of platelets in pure plasma and in plasma containing 0.3 per cent. of Witte's "peptone." All operations were conducted in paraffined vessels, without exposure to the air. Temperature 20° C.

Nos. of experiments.	Medium containing platelets.	State of digestion of donor.	Times required for clumping and partial lysis of platelets.		Times required for the complete lysis of platelets.		Times required for the complete gelation of the plasma.	
			Minimum.	Maximum.	Minimum.	Maximum.	Minimum.	Maximum.
1 to 5	Pure plasma	Fasting	mins. 25	mins. 35	mins. 38	mins. 40	mins. 42	mins. 47
6 to 10	Pure plasma	After meat meal	16	24	19	22	25	32
11 to 16	Pure plasma	After meal of rice	26	39	36	43	41	49
17 to 22	Plasma containing 0.3 per cent. of "peptone"	Fasting	40	80	55	64	80	103
23 to 26	Plasma containing 0.3 per cent. of "peptone"	After meat meal	23	25	27	36	33	42
27 to 32	Plasma containing 0.3 per cent. of "peptone"	After meal of rice	40	78	51	62	79	96

Table II.—Illustrating the Minimum and Maximum Times (in minutes) required for the clumping and lysis of both unwashed and washed platelets when removed from the plasma and either transferred to the medium named or returned to the plasma. Temperature 20° C. The "peptone" used was dissolved in 0.875 per cent. NaCl.

Nos. of experiments.	Condition of platelets.	State of digestion of donor.	Medium containing platelets.	Times required for clumping and partial lysis.		Times required for complete lysis.	
				Minimum.	Maximum.	Minimum.	Maximum.
33 to 38	Unwashed	Fasting	0.875 per cent. NaCl	mins. 16	mins. 19	mins. 19	mins. 23
39 to 43	Unwashed	Fasting	Plasma	26	32	33	38
44 to 48	Unwashed	Digesting meat	0.875 per cent. NaCl	9	11	13	16
49 to 52	Unwashed	Digesting meat	Plasma	12	15	17	22
53 to 57	Unwashed	Fasting	0.3 per cent. Witte's "peptone"	27	31	32	44
58 to 61	Unwashed	Digesting meat	0.3 per cent. Witte's "peptone"	18	24	21	26
62 to 65	Washed	Fasting	0.875 per cent. NaCl	12	13	14	17
66 to 69	Washed	Fasting	Plasma	17	22	20	27
70 to 73	Washed	Digesting meat	0.875 per cent. NaCl	8	10	9	14
74 to 79	Washed	Fasting	0.3 per cent. Witte's "peptone"	11	13	13	16
80 to 84	Washed	Digesting meat	0.3 per cent. Witte's "peptone"	10	11	12	13

Discussion.

The experiments recorded in Tables I and II show that platelets suspended in undisturbed plasma are more resistant to clumping and lysis than those deprived of association with that fluid. This is explicable by the finding that undisturbed plasma, when uncontaminated by the débris of platelets, is a relatively stable fluid (7). It appears established that films of such plasma protect platelets against clumping and lysis.

A comparison of experiments 1-5 with those numbered 17-22 shows that a concentration of Witte's "peptone" just sufficient to inhibit the clotting of human blood (0.3 per cent.) augments the stabilising influence of plasma on platelets, and it is evident from the results obtained in experiments 62-84 that a like concentration of "peptone" has hardly any action on platelets which have been deprived of plasma by washing in isotonic saline. It is also patent, from experiments 17-32, that the anticoagulant action of "peptone" on blood freshly shed into paraffined vessels continues after the complete disintegration of platelets.

From these observations, the present writer rejects the conclusion of Mills (9) that the anticoagulant action of small amounts of "peptone" on freshly drawn blood is solely due to the prevention of the lysis of platelets. It appears that the principal influence of minute quantities of "peptone" on shed blood is restraint of changes in the plasma, and that this reaction, by temporarily preventing the disintegration of platelets, assists in the inhibition of blood clotting. The apparent discrepancy of these conclusions with the observations of Mills and his co-workers (9, 14) on the action of "peptone" on citrated and "re-calcified" plasma is explicable by the finding (by Pickering and Hewitt (11)) that citration and "re-calcification" disturb plasma complexes and render them hypercoagulable even *in vivo*. It should also be noted that in the experiments of Mills and his associates the action of the air on plasma and platelets was not eliminated.

The observations of Chio (12) and of Baumberger (13) suggest that an intimate relationship exists between the pressures of oxygen and carbon dioxide in the plasma and the maintenance of the fluidity of the blood. The observations here recorded indicate that the escape of gases from the plasma produces disintegration of platelets and so facilitates the inception of blood clotting. They also imply that the stability of platelets is dependent on the pressures of these gases in the plasma.

The discovery of Mills and his colleagues (14) that platelets obtained during

the height of digestion of a meat meal both clump and disintegrate more rapidly than those withdrawn during either fasting or the digestion of a meal of rice, is confirmed. But the differences here recorded in the resistance of platelets to clumping and lysis under these conditions are much greater than those recorded by Mills. This may be attributed to the fact that in the earlier experiments contact of the air with platelets was not avoided, with the consequence that changes in the speeds of clumping and lysis were partly masked.

The recognition of the lowering of the resistance of platelets to clumping and lysis during the height of digestion of protein may throw some light on certain harmful reactions which sometimes follow the transfusion of blood that has satisfied the usual tests for compatibility. Brem and his associates (15) observed indications of incompatibility following the transfusion of the blood of donors who were at the height of digestion of a meat meal, and that the blood of the same donors does not produce these effects when obtained during fasting.

Summary.

- (1) Undisturbed plasma protects platelets against clumping and lysis.
- (2) A concentration of Witte's "peptone" just sufficient to inhibit the clotting of human blood augments the stabilising influence of plasma on platelets.
- (3) The anticoagulant action of "peptone" on plasma shed into paraffined vessels continues after the disintegration of platelets.
- (4) The principal influence of small amounts of "peptone" on blood freshly shed into paraffined vessels is the restraint of changes in the plasma. This reaction, by delaying the disintegration of platelets, assists in the inhibition of blood clotting.
- (5) Exposure of the plasma to the air produces disintegration of platelets. It appears that the stability of platelets is dependent on the pressures of gases in plasma.
- (6) The observations of Mills and his assistants that platelets withdrawn from circulation during the height of digestion of a meat meal clump and disintegrate more rapidly than those obtained during either fasting or after the ingestion of a meal of rice, is confirmed.

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Observations on Electrical Stimulation of the Cerebellar Cortex.

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(PLATES 33-35.)

Introduction.

In recent years Miller (8) and Fulton (6) among other authors have produced valuable reviews of the question of cerebellar function. Of primary importance in investigating cerebellar activity by stimulation of its cortex is the fundamental observation of Sherrington (12) that faradisation of the rostral surface produces ipsilateral inhibition of decerebrate rigidity. Latterly this observation has been confirmed and amplified by Bremer (1) and by Miller and Banting (7) among others. Electrical stimulation of the anterior lobe inhibits posture in ipsilateral antigravity muscles and augments the posture of contralateral antigravity muscles. Excision of the anterior lobe produces

spasticity of antigravity muscles on one or both sides. In the pigeon, which lacks a pyramidal tract, Bremer and Ley (2) have obtained similar results by excision of, or by stimulation of, the cerebellar cortex. Pollock and Davis (10) have produced similar results in cats by rendering anæmic the anterior part of the cerebellum at the same time as the precollicular neuraxis. Rademaker's animals, from which the entire cerebellum had been ablated, were observed for many months (11) and throughout that time hypertonicity of antigravity muscles was a prominent feature of their symptoms. More recently, Miller and Laughton (9) have described the result of stimulating, not the cerebellar cortex, but the nuclei themselves, which were exposed by ablation of the overlying nerve tissue.

We proposed therefore to extend these observations by use of a dead-beat recording mechanism, namely, the Sherrington myograph.

Method of Experiment.

The animals used were cats. Under profound chloroform-ether anæsthesia and after ligation of the carotid arteries in the neck a trephine opening was made in one temporal fossa and carried across the midline to the opposite fossa. With the upper surface of the cerebral hemispheres thus exposed, access to the brain stem was facilitated and the level of transection could be accurately gauged to be in front of the anterior colliculi and slope forwards. The preparations therefore showed posture of both flexor and extensor muscles, being rather greater in the latter group for the first hours after decerebration. After decerebration, the opening was enlarged posteriorly and the tentorium cerebelli ablated, thus giving wide exposure of the cortex cerebelli.

For electrical stimulation, we used bipolar electrodes 2 mm. apart, fed from a Berne coil (coreless) with 2 volts and 2 ohms resistance in the primary circuit. The cerebellar cortex was dried carefully before each of our observations with a pledget from saline solution at 37° which was well squeezed out. Escape of current under such conditions on the surface of the cerebellum did not exceed 1.5 mm. from the electrodes as judged by the reactions of a frog nerve-muscle preparation laid upon the cortex when the secondary coil was 6 cm. distant from the primary (in this experiment 4 volts and 0.08 amp.). We noted in this connection that when a too-hot pledget of wool was applied to the rostral region of the cerebellum, inhibition of antigravity muscles ensued in a manner indistinguishable from that occurring under electrical stimulation. The significant testimony of this result from non-electrical stimuli has been already emphasised by Bremer on several occasions. On occasion the preparation's

reactions to cerebellar stimulation have been studied by clinical inspection, that is, with all peripheral nerves intact and without myographic recording. When recording has been undertaken, the recording apparatus has been the double shadow myograph with isometric levers of high vibration frequency (1000-1200 d.v. per second) combined in the same optical system as a string galvanometer of the Cambridge Instrument Co. with double string case.

A variety of muscles have been used, after careful immobilisation by nerve or tendon resection of all other muscles in the same limb and opposite limb. When two muscles have been observed at one time in the double myograph, the two selected have been one a flexor, the other an extensor, *e.g.*, M. Brachialis Anticus and M. Triceps Brachii (with the scapular head resected) in the fore-limb, M. Quadriceps and M. Semitendinosus in the hind-limb. In general, the cerebellar cortex of the ipsilateral side has been stimulated.

Inhibition of Extensors.

Under scrutiny by the high speed myograph, well defined inhibition of extensors can be effected by a rapid repetitive stimulus applied to the cortex cerebelli, though it is usually late in appearance in the myograph record, slow in development and brief in after-effect. These characteristics are marked when the background is the reflex of crossed extension, figs. 1A and 1C (Plate 33),

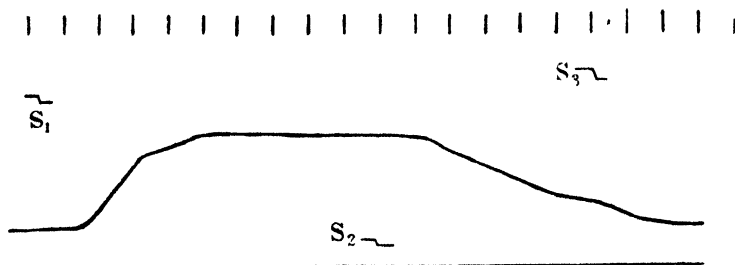


FIG. 1A.—Quadriceps. Crossed sciatic nerve stimulated at fall of S_1 , 9 cm. coreless class coil. At fall of S_2 , point A on map of cerebellum, stim. coreless Berne coil 8 cm. S_2 , crossed stim. off, cerebellar stim. continuing. Rate of stim. of cerebellum = 48 break shocks p.s. Rate of crossed stim. 49 break shocks p.s. Time 0.1 second marked at top of record.

and are contrasted with the events in fig. 1B which shows the sudden onset and rapid fall in tension associated with the inhibition evoked in this reflex by stimulation of an ipsilateral nerve. A long after-effect is also well-known to occur under these latter conditions. The inhibition evoked from the cerebellum similarly affects the stretch reflex after a comparatively long latency,



FIG. 1B.—Same preparation, same crossed stim. At fall of S_2 , ipsilateral sciatic nerve stimulated, Berne, 12 cm.

and thereafter the decline in tension is almost as slow as when the background is crossed extension. The cerebellar effect is therefore an inhibitory recruitment.

Slow rates of stimulus, of from 8 to 13 break shocks a second, reveal several features of the inhibitory recruitment. It is unusual for the first few break shocks of a weak stimulus series to produce any effect in the myograph record, but each of the later shocks produces a slight fall, which is arrested by the

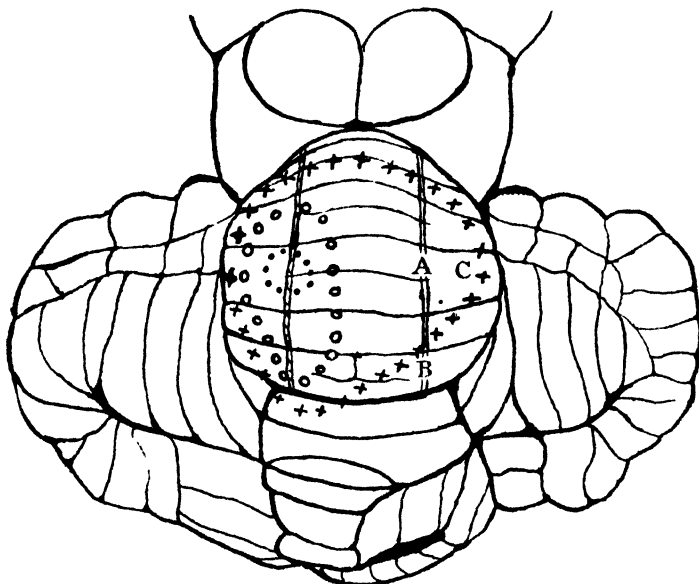


FIG. 1D.—Map of Cerebellar Cortex.

Dorsal aspect of cerebellum of cat. A, B and C mark points stimulated as indicated in the legend for each figure. The double lines crossing the vermis represent the lateral superior cerebellar vein. Dots circumscribe an area from which elbow flexion was elicited in one preparation at 12 cm. coil (Berne coreless 2 v. in primary). Circles circumscribe area when coil at 10 cm., and crosses area when coil at 3 cm. (see p. 532).

central discharge occurring before the next shock (fig. 2A, Plate 33). When the background excitation is powerful the action currents of the galvanometer record at first show no effect, but, as the inhibition becomes more profound, each break shock produces an interval of quietness in the action currents, proceeding to intervals of increasingly more complete silence (fig. 2B, Plate 33). During the progress of recruitment of inhibition the true latency of effect of each stimulus in the series can be measured to the onset of the period of string quietness and is found to vary a little above or a little below 25σ . Each stimulation to the *cortex cerebelli* therefore exercises a brief damping effect on the spinal discharge of short latency and short total effect. Its latency is, however, longer than that of the excitation of the final motor neurone elicited by electrical stimulation of the *cortex cerebri* which Cooper and Denny-Brown (4) have found to be about 14σ (ether), without using strychnine.

The brevity of effect of a single stimulus in a slow series and the slow development of the inhibition from a rapid series may be in part due to the weakness of stimulus used, but increase in strength of stimulus (with consequent greater liability to escape) does not alter these features although tending to make the subsequent course of the inhibition more profound. The rapid recovery from the cerebellar inhibition is, we think, related to the common occurrence of rebound after the inhibition noted by Bremer (1). We have also found terminal rebound to be a common occurrence, and confirm Bremer in that the terminal rebound is much increased by repetition of the stimulus period (facilitation of rebound).

The tendon jerk proved valuable for an analysis of the effect on extensor posture. In fig. 3A, Plate 33, the inhibition from the cerebellum will be seen to cause the usual slow fall in tension. Even allowing for that fall the height of each individual reflex diminishes gradually. Concomitant with this change and presumably the cause of it, is a lowering of the "point of inflection" of the jerk (6). At first glance, the less steep ascent of the jerk suggests that there is a change in latent period, but we have failed to find any change in latency, as measured from the beginning of the tap to the beginning of the action current, nor is the total duration measureably prolonged. But it is not improbable that there is some slight increase in the "synapse time" of the jerk under cerebellar inhibition, which might have been detectable had we used a long rapidly moving photographic film allowing a prolonged stimulation of the cerebellum to be observed. The silent period in the string record which follows the action current of the jerk shows a gradual lengthening. Thus after a series of fourteen consecutive jerks (about 500σ apart) the "silent period"

was 125 σ —a notable lengthening in comparison with the normal of 55 σ (fig. 3B, Plate 34). The resting tension during this time had fallen from 1.0 kg. to 0.36 kg. Recovery of the jerk after inhibition occurs rapidly. The fifteenth jerk of this series fell 220 σ after the cessation of inhibition. Its "silent period" lasted 75 σ , at a time when the resting tension had risen to 0.5 kg. The next jerk, 400 σ later, had a silent period of 60 σ and a resting tension of 0.6 kg. From this and other similar records we conclude that the duration of the silent period, before, during, and after inhibition, bears to the resting tension of the muscle some clear relationship, but which is rather too indefinite at closer inspection and too variable to be plotted graphically.

The progressive lengthening of the silent period during the recruitment of inhibition and its shortening with the appearance of recovery from the inhibition is an indication of the change of state of the stretch excitation at those units which respond in the tendon jerks and reveals the corresponding change in the static stretch reflex. The tendon jerks often lose tension only late in the inhibitory period, after the lengthening of their silent period, and this is regarded as further evidence of the slow progression of the inhibitory state at these units, which thus allows differentiation between the synchronous afferent volley of the tendon jerk and the asynchronous stretch background.

After inhibition it may be observed that the jerk shows a gradual increase to its original amplitude, at a time when the stretch background has increased by a very small and almost imperceptible amount. This denotes a state of greater wakefulness, following stimulation of the units involved in stretch reflex, a state to which the name of "latent successive induction" (5) has been given.

On occasion, the tendon jerk yields very readily to inhibition, and may be almost entirely extinguished after 0.9 second of tetanic stimulation of the cerebellar rostrum. During this time the resting tension of the muscle falls *e.g.*, from 750 to 450 grms. That this is not directly related to the suppression of the jerk is shown by the fact that 0.3 second after the cessation of stimulation, and with the resting tension still at 450 grms., a jerk of 700 grams tension can be elicited—as large as the control jerk. In other words, and as shown by other ways, the kinetic stretch reflex (jerk) is less susceptible to impairing influences than the static stretch reflex (posture).

Excitation of Flexors.

Recently Miller and Laughton (9) have published an account of the excitation of flexor muscles which reciprocates with the inhibition of extensor muscles

when the cerebellar nuclei are themselves directly stimulated with a stigmatic electrode. We found it possible to excite ipsilateral flexor muscles to activity by weak stimuli of the rostral region of the cerebellar cortex with bipolar electrodes at points which were far removed from cerebellar nuclei.

For example, fig. 4, Plate 34, shows a contraction of the recruitment type in *M. Brachialis*. Before the application of any stimulus, the tonic posture of the flexor in the precollicular preparation is evidenced by typical asynchronous rhythm in the string record. At the onset, there is a slight inhibitory fall accompanied by "silence" in the string. Then there develops an irregular recruiting response, which climbs to a considerable tension (1.4 kg.) followed by a rebound after the cessation of the stimulus. The plateau of rebound turns sharply into a slow descent to the base line. When this stimulus is repeatedly turned on and off, facilitation of the flexor excitation occurs and every feature of the response is made more prominent by repetition. The early inhibitory drop becomes marked and the latent period of the recruiting rise lengthens progressively.

In fig. 5 there is recorded the excitation of a flexor from a low initial tension so that there is no apparent inhibition. The response begins after a latent period of 2.8 seconds, during which 26 break shocks were applied to the cerebellar cortex. The rise is gradual, but shows the rhythm of excitation. Irregularities in the rise are exaggerated steps in the rhythm. After climbing for 4 seconds, the reflex declines a little and at the end of excitation subsides

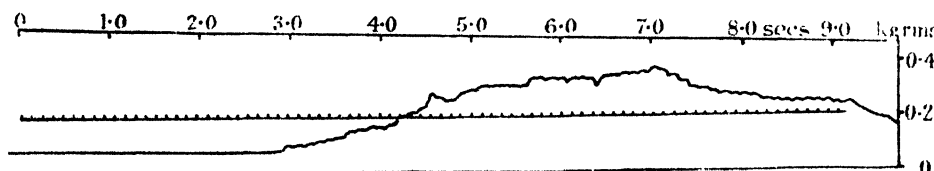


FIG. 5.—*Brachialis anticus*, same preparation as in fig. 4, slower plate, time in seconds. Vibrations of time-marking signal show onset and rate of cerebellar stimulation, Berne coil coreless, 2 cm., spot B, 9 break shocks p.s. Stimulation stops when signal stops.

showing marked after-discharge. During that period of 2.8 seconds excitation is in a latent process of accretion at the nerve centres, comparable to the "summation period" of excitation produced by stimulation of the cerebral cortex (4), until finally it reaches a supraliminal value and the final motor neurones discharge. A similar response from the same preparation was elicited with a much shorter latent period, namely, after only 3 break shocks.

When excitation at the flexor centre has been raised above its supraliminal

value by previous stimulation of a nerve of the ipsilateral limb, cerebellar stimulation can be seen to affect the string galvanometer record immediately, by the occurrence of nodding (fig. 6, Plate 34). Yet the cerebellar stimulation does not succeed in adding to the number of muscle units engaged, since there is no change in muscle tension and the regular arrival of series-stimuli from one source interferes with the regularity of discharge produced by the series-stimuli from the other source. In other words, since the activity of those units which are engaged in the pre-existing flexor response is already maximal, the cerebellar stimulation cannot contract them further.

Inhibition of Flexors.

It is not uncommon to record inhibition of flexor activity, which, after an initial steep downward slope, continues to lapse further during the persistence of the stimulation and does not show a late rise of excitation as described in the preceding section. The features of such a record are in general indistinguishable from those elicited by mild spinal inhibition (fig. 7). The latent

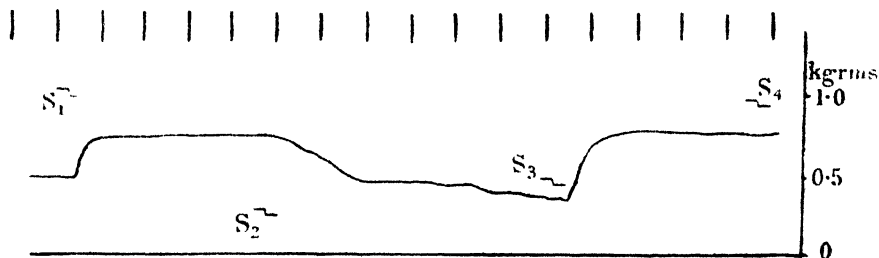


FIG. 7.—Brachialis anticus. Ipsilat. median nerve stimulated at S_1 , coreless class coil 14.5 cm. At S_2 , spot A of cerebellum stimulated, coreless Berne 4 cm. off at S_3 . Median stimulation off at S_4 .

period of the myograph fall inclines to be long, *e.g.*, 60 σ with weak cerebellar stimulation, that is, when the first drop is only gradual. When the stimulation is stronger and the drop more abrupt (fig. 8) the latent period is less, *e.g.*, 25 σ , although both or either of these figures may be reduced, if it is known how many individual stimuli, the time apart of which is rather more than 20 σ , fall after the opening of the key. There are occasions when the cerebellar stimulation does not provoke rebound and those flexor units which have been inactivated by the previous inhibition remain out of action in spite of the continuance of stimulation to the nerve of the ipsilateral limb, although fig. 8 is not a good example of this, for the exciting nerve is itself a "mixed" nerve, as evidenced by the rebound after cessation of its stimulation.

Nevertheless, the long-lasting effect of a preceding inhibition is well known as a purely spinal phenomenon.

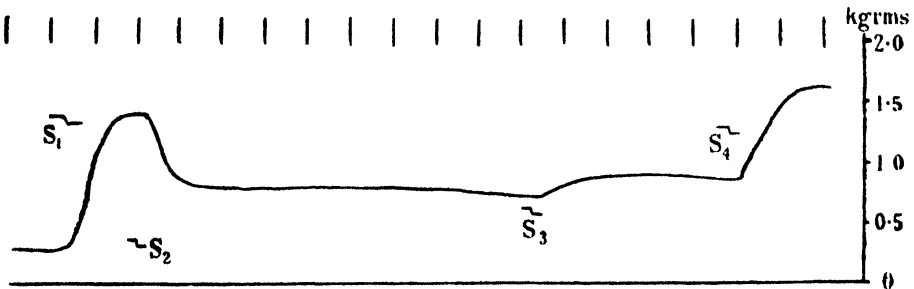


FIG. 8.—Brachialis anticus. Same preparation as fig. 7. At S_1 , ipsilateral ulnar nerve stimulated students' coreless coil 10 cm., 48 break shocks p.s. At S_2 , spot B, stimulated, coreless Berne 2 cm., 49 break shocks p.s. At S_3 , cerebellar stimulation off at S_4 ulnar nerve off.

When a stimulation at a slower rate than this is used, with stimuli 80 σ apart, against a myographic background which it neither excites nor inhibits, the string record shows interesting changes (fig. 9, Plate 33). Before the incidence of the stimulus, the string record of the flexor shows the asynchronous rhythm which is characteristic of a muscle in posture. 10 σ after the stimulus has been delivered on the cerebellar cortex, the string record shows a large single vibration such as that which accompanies muscular activity. This lapses after 5 σ and is followed by one or two small vibrations for about 12 σ . Then for 20 σ the string shows complete quietude, indicative of inhibition. This is followed for 20 σ by one or two large vibrations of the string, which suggests rebound. Then the string settles down into its original state of showing the small vibrations of posture. In brief, a single break shock to the cerebellum has a double effect on the flexor centre, excitation and inhibition. Sometimes with this series of stimuli there appears to be an excitation of the flexor, which reveals itself not as the increased size of the marked wave of excitation immediately after the break shock, but as a gradual increase in the waves of rebound after each period of silence in the string. Excitation in such a case as this amounts in fact to a series of rebounds, rapidly repeated and of increasing size, for the inhibition from each break shock here precedes the corresponding excitation.

Excitation of Extensors.

Myographic analysis of the inhibition of extensors ensuing from stimulation of the cerebellar cortex, revealing as it does an inhibitory process which is very slow in onset and slight in after-effect, followed by frequent rebounds

when no previous excitation has been evident, leaves the impression that the inhibition is the result of a conflict between concurrent inhibition and excitation. It is therefore not altogether surprising to find further evidence of excitation occurring during the stimulus, for delayed onset of inhibition and rapid terminal rebound have come to be regarded as signs of a concurrent mixed excitatory and inhibitory effect in limb reflexes. In fig. 10A the cerebellar cortex is stimulated intercurrently during the response of *M. Supraspinatus* to the ipsilateral median nerve of the upper arm, which is well known to be "mixed." The first part of such a response to cerebellar stimulation is an outburst of excitation (latency 40 σ) followed by a fall to a lower level than that of the control observation. But even in that fall there is an excitation, for the level falls lower still at the cessation of cerebellar stimulation.

This cerebellar stimulation produces strong responses from the *M. Supraspinatus* of the opposite side. When the cerebellar stimulation is made stronger (fig. 10B) the excitatory outburst is smaller and has a longer latency

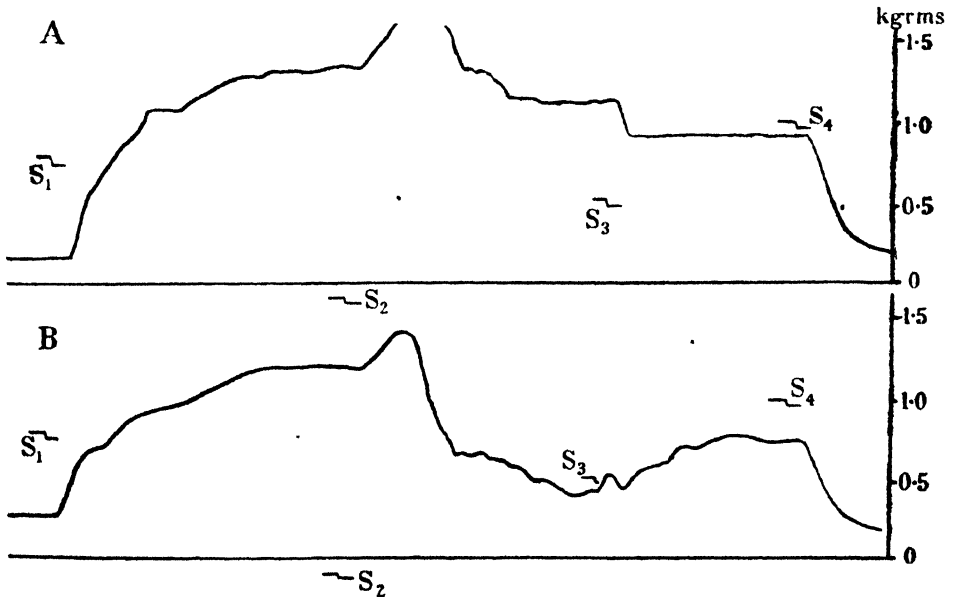


FIG. 10A.—*M. supraspinatus*. At S_1 ipsilateral median nerve of upper arm stimulated coreless student's coil 9 cm. At S_2 point A of cerebellar stimulation, coreless Berne coil 10 cm. (4 volts). At S_3 , cerebellar stimulation off, at S_4 , median stimulation off.

FIG. 10B.—Cerebellar stimulation made stronger, 7 cm.

(80 σ) and the ultimate inhibition is more profound, carrying with it little obvious excitation but only a small rebound at the end. When the cerebellar

stimulation is stronger still (fig. 10C), there is very little preliminary excitation, but only the steep inhibitory drop of the same latency as before (240σ).

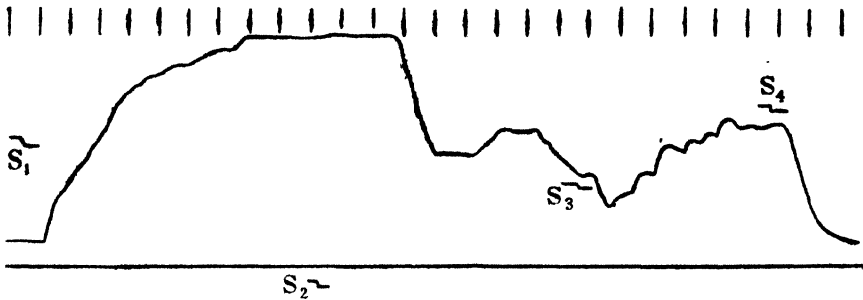


FIG. 10C.—Cerebellar stimulation made stronger, 4 cm.

Excitation is evident in the trough of inhibition and there is a further fall of tension when the stimulus is stopped (post-inhibitory notch).

Our observations are then precisely those which might be observed with any peripheral nerve and define the cerebellar cortex in the region where we have stimulated as a structure where similarly "mixed" functions are subserved. The increase in inhibitory effect as the stimulus is strengthened suggests a preponderating influence of inhibition from the cerebellar cortex, while the diminishing effect of excitation as the stimulus is strengthened controls the possibility of that effect being due to gross "escape of current" to deeper structures or to the cortex of the other side. Besides this type of early excitation of extensors, which appears from analysis by the double myograph to be the reciprocal counterpart of the early inhibition seen in the flexors, there is frequently encountered a recruiting type of excitation with terminal rebound

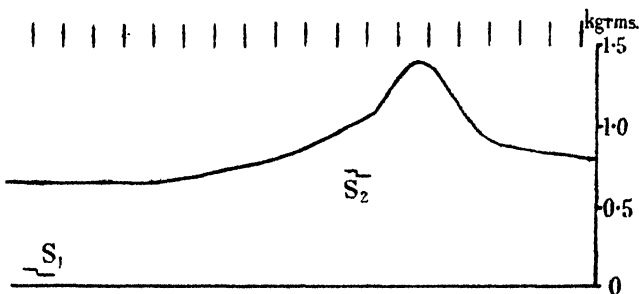


FIG. 11.—Supraspinatus. Between S_1 and S_2 point A of cerebellum stimulated, coreless Berne coil 9 cm., 49 break shocks p.s.

(fig. 11). A feature of the rebound is that it may have a latent period as long as 60σ .

When the cerebellar stimulation is made intercurrent in a background of crossed extension (fig. 12, Plate 35, upper record), it is seen that as with the ipsilateral of *M. Supraspinatus* it may add further to an already rising plateau, but that this later gives way and remains at a steady level of tension at a time when the control record (not shown) is still climbing. When the cerebellar stimulation ceases, and in spite of the continuance of the crossed stimulation, the plateau of tension falls abruptly and the string ceases to vibrate, factors evident of inhibition. This terminal fall in tension (the post-inhibitory notch) suggests that some of the preceding reflex excitation has been due to the stimulus from the cerebellum which has just ceased.

Again a series of break shocks repeated slowly has proved valuable in analysis of the excitation of extensor muscles. In fig. 13, Plate 34, there are seen to be periods of silence in the string 28 σ after break shock, and lasting 30 σ . Separating the periods of silence there are series of action currents often beginning with a large wave. As in the case of the flexor this is rebound, following inhibition. In fig. 14, Plate 35, however, the extensor muscle's record is almost uniformly active, but shows an early action current after the delivery of the break shock. These appear to be the action currents of excitation only without the manifest inhibition which in fig. 13, Plate 34, depresses the string movements for 30 σ . It is obvious that rapid repetition of break shocks will produce an overlap of the inhibitory effect. In fig. 12, Plate 35, the excitation breaks through such a fusion of brief inhibitory periods and a recruiting excitation develops. The short lasting nature of each break shock's inhibitory period is reflected in the short latency of rebound following the rapidly repeated stimulus series, *e.g.* fig. 1C, Plate 33.

Reciprocal and Simultaneous Action of Flexor and Extensor.

Although perfect reciprocity of action between flexor and extensor has been the more frequent in our observations, this is not invariable, and simultaneity of action has been often recorded. In the record (fig. 9) described in a previous paragraph, the string record of the extensor muscle showed marked activity during the inhibition of the flexor, there being one or two small but deliberate vibrations in the extensors string record at that period. Their smallness in comparison with those of the preceding flexor excitation, and their onset some 6 σ after the flexor's string has been silent, are features which call to mind the extensor's greater "inertia" in other reflex activities.

In fig. 12, Plate 35, both flexor and extensor muscles are excited by stimulation of the median nerve of the other fore-limb. During the response the cerebellar

cortex is stimulated. Within 20 σ the flexor is inhibited (the rate of stimulation being about 50 break shocks per second). This is followed by the slow rise in the myograph which was described for cerebellar stimulation alone (fig. 4, Plate 34) and which was more marked in that figure, and in this preparation also in records made before and after fig. 12. Later, there is rebound concurrent with the inhibitory drop in the flexor, the extensor muscle shows a rise and later a fall, at a time at which the flexor's tension is increasing. 80 σ later than the onset of rebound in the flexor, the extensor shows a decline in tension. This reciprocity of action between flexor and extensor is not greatly different from that described in the simplest form in the spinal animal. In other records reciprocity has been less marked and both muscles have rebounded together, the flexor's rebound preceding the extensors by some 20 σ , though the latter when it has occurred has been larger and longer lasting.

In fig. 15 the muscles again are both relaxing in response to the stimulus. At the onset of each period of stimulus the tension of both muscles falls. Between the inhibitory periods, both rebound. The facilitation of the rebound previously described for the flexor is seen also in the extensor, but is more sluggish in development and less marked in degree.

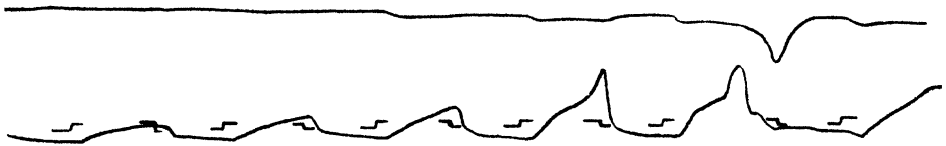


FIG. 15.—Upper myograph, triceps, lower, brachialis anticus. Coreless Berne coil 2 cm., 49 break shocks p.s. used to stimulate point C of cerebellum. Lower signal up = stimulation off, down = stimulation on. Each period of stimulation lasts about 1 second.

With single break shock stimuli spaced 80 σ apart the simultaneous action can be seen again in fig. 9, Plate 33, taken with postural muscles of which the tension is unaffected. During the second phase of the response after the single break shock, when the flexor's string record is quiet, the extensor's string is vibrating, but those vibrations last less long than the quiet period in the flexor's string and cease at a time when the flexor's string is still motionless. In other words, the muscles show simultaneous inhibition for a brief period of 8 σ . In a record, fig. 16, Plate 35, taken 2 hours earlier from the same preparation, the passage of events was rather differently timed, for in that record the

extensor's string vibrated during all the quiet period of the flexor's string which was here very much shorter and ceased to vibrate when the flexor's quiet period was over. The general tendency appears then from our experiments for the reflex discharge of the flexor and extensor centres in response to each stimulus member of the series to be reciprocal. As a rule, with rapidly repeated stimuli (*e.g.*, 50 break shocks per second) the flexor tends to be inhibited first and then to recruit to a low plateau, while the extensor behaves reciprocally and is excited and later inhibited.

Effect of Cerebellar Stimulation in the Non-denervated Preparation.

When the cerebellum of a decerebrated, but otherwise intact, animal is stimulated, and the reactions are observed by clinical inspection, inhibition of all antigravity muscles is most apparent. The cerebellar effect does not establish itself until the anæsthetic has passed off and even then it appears in the neck and fore-limbs some time (*e.g.*, 1-1½ hour) before it is found in the hind-limbs and tail. The gradual onset of decline of the decerebrate rigidity, when it occurs, is in marked contrast to the abrupt decline of rigidity evoked by stimulation of a spinal nerve, and calls to mind the slowly developing variations in limb posture evoked in the neck and labyrinth reflexes. When the level of decerebration is well pre-collicular, it is common to find that cerebellar stimulation elicits a sharp flexion of the elbow joint, flexion (dorsi-flexion) of the wrist, abduction and extension of the digits and protrusion of the claws. The attitude of the fore-limb is just that which is found in the "rampant" animals of heraldry. After and sometimes during the stimulation, the limb "strikes" by the elbow becoming extended, the wrist and fingers flexed. This sequence of events seems to be only a "slow-motion" of the "Laufbewegungen" familiar in precollicular preparations. In fig. 17, Plate 35, there is a myograph record of this action. After prolonged cerebellar stimulation the flexor muscle (*Brachialis Anticus*) recruits with rhythmic and rather irregular steps. During this time, the extensor muscle slackens very slightly. The flexor's contraction eventually rushes to a pinnacle of high tension and the string is very active. Although the beginning of the flexor's drop is out of sight, it is evident from the string that this has a sharp turn, for the string movements rapidly diminish in size and number until the flexor's tension falls to nil, with complete quiescence in the string record. In perfect reciprocity with the fall in the flexor's tension, the extensor (*Triceps*) rapidly gains a high degree of tension, with vigorous string movements. These movements are all executed during the continuance of cerebellar stimulation, and are pre-

cisely those which correspond with the striking or clawing movements observed by us and by others under the same conditions of experiment.

In one case with the usual strength of stimulation (3 cm.) a very forcible and rapid flexion of the elbow was obtained over a large area of the cerebellar cortex extending well over to the contralateral side (see Map, fig. 1D). Decreasing strength of stimulation (10 cm.) diminished area for flexion which was now confined to ipsilateral side. Further diminution to 12 cm. greatly restricted the excitable area and the intensity of the response. It seems unlikely that the larger effective area of the stronger stimulation was due to spread of the stimulating current to this very low threshold region, as the increase is much more across the median line than laterally. Probably the movement of elbow flexion was represented over the whole area, being especially prominent in the above-mentioned low threshold region, from which it was alone elicitable in less favourable preparations.

Discussion and Conclusion.

The most constant effect of stimulating the cerebellar cortex is inhibition of antigravity muscles. The type of inhibition differs from spinal inhibition only in speed of onset and development. The ultimate locus of its incidence is the final motor neurone. The more gradual onset of the inhibition need not be because the nerve impulses have to traverse many synapses, since the latent period of the first trace of inhibition may be surprisingly brief, especially in flexors. It is as though each connection from the cerebellar cortex to the final motor neurone is almost direct, and, given time, the neurone's activity may be entirely inhibited even in the face of a strong crossed extensor response. The sharp rebound, which is a prominent feature of this inhibition and has been described by earlier workers, is the result of an excitatory process from some cortical units concurrent with an inhibitory from others, and is elicited by the same stimulus. It is difficult to obtain anything but a mixed effect from the cortical stimulus, whether in flexor or extensor. This may well be taken to indicate the microscopical admixture of elements in the cerebellar cortex exerting opposite effects on the lower motor neurones. The area of cortex from which effects were obtained extended not only over the anterior lobe, but also more posteriorly on the vermis and on the lateral hemisphere, although in these latter regions an escape of current to the cerebellar nuclei and fibre bundles cannot be so carefully controlled. The effects here described concern always the muscles of the same side. The degree of excitation and of rebound appears to be greatest in the region of the vermis of the anterior lobe. The effect on

that side is the stronger, but there is an effect on the contrary side. This is less marked and is mainly of an opposite nature, but there is mixed with it an effect similar to that obtained on the stimulated side.

The striking or clawing movements which we have observed and recorded, and which have been observed by others, need not be any other than the normal movements of the precollicular preparation, here at the one time aided in initiation and slowed in execution by the cerebellar stimulus. The lower centres in other words are in an unstable state, tending to discharge that reflex-type, and by the cerebellar stimulus are brought to surpass the liminal value for discharging the latent reflex, and perform it slowly, as though "by numbers." Thus the cerebellar cortex calls forth latent reflex events at the spinal neurones and then underlines and makes them more emphatic. Every reflex response in a muscle with its afferent supply intact is the sum of simultaneous excitatory and inhibitory processes at the spinal neurone. The cerebellum acts by reinforcing one of these processes at the expense of the other. In this way there can be found excitation and inhibition of flexor and extensor spinal reflexes, such as (1) the flexor "withdrawal" reflex, (2) the crossed extensor reflex, (3) the ipsilateral extension reflex of *M. Supraspinatus*, and (4A) the stretch reflex, static or postural, (4B) kinetic or tendon jerk. The nature of the excitation and inhibition from the cerebellum does not appear to differ in any essential mechanism from that found in other reflexes.

So far as it is possible to speak of a "point" at all where bipolar electrodes are used (or even monopolar), as Graham Brown and Sherrington have pointed out (3), it is evident that every "point" or spot has underneath it nerve units of four tendencies, inhibition and excitation of extensors and inhibition and excitation of flexors. Since all kinds are stimulated together by our methods the effect of stimulation is mixed and unstable, and this mixed cerebellar effect is superposed on a more stable taxis, the spinal reflex. The response of the muscle may therefore be inclined and reinforced in one or two directions. The reversibility and ambiguity of a cerebellar point is frequent and calls to mind the well-known reversibility of points on the cerebral cortex. On one occasion, for instance, one point within a period of 15 minutes gave "inhibition of flexor" — "flexor rebound, nothing in extensor" — "inhibition of flexor and extensor." The anatomical structure of the cerebellar cortex allows natural reflexes to select discrete, functionally similar, cortical units with harmonious motor effect. Postural reflexes on account of their ready tendency to yield to stimuli of a more remote origin than themselves form the most favourable background upon which to demonstrate cerebellar effects. This need not be taken to imply more

than a partial postural function for the cerebellum in those regions of the cortex from which we have obtained positive results.

Summary.

1. In decerebrate cats with precollicular section the cortex of the anterior lobe of the vermis and the median part of the cortex of the lateral lobes of the cerebellum have been stimulated with induced currents.

2. When so stimulated the cerebellar cortex of these regions imposes a modification on pre-existing spinal reflex acts or states, but does not directly initiate them.

3. Reflex inhibition and excitation of extensor muscles and reflex inhibition and excitation of flexor muscles have been found. It is probable that this result depends on the stimulation concurrently affecting intermingled nerve units of varying function in the cerebellar cortex.

4. The predominant feature of a rapid stimulus series is inhibition of reflexes of the extensor group of the same side as that stimulated. This is followed by a post-inhibitory rebound.

5. Apart from this inhibition and the rebound resulting from it, the cerebellar cortex may exercise a pure excitatory effect of short latency on the reflexes of the extensor group. This occurs with weak series stimulation.

6. In a slow series of break shocks, one single shock reveals both in extensor and flexor a biphasic response, *i.e.*, inhibition followed by excitation or vice versa. When excitation precedes the inhibition, it cannot be a rebound. All processes are of shorter latency in the flexor.

7. Responses to a rapid repetitive series are the resultant of the relative values of these combined processes.

8. The relationship of activity of extensor muscles to flexors of the same side is usually reciprocal, but it may be simultaneous.

9. Flexor and extensor muscles of the contrary side are made active usually in the phase opposite to that of corresponding muscles of the stimulated side, but to a much less degree.

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DESCRIPTION OF PLATES 33-35.

PLATE 33.

Time in plate figures 0.02 second, and string tension 2-3 mm. per 1 m.v. (mag. 390 ×.)

FIG. 1C.—Supraspinatus. M = myograph record. Sg, string record. Between S₁ and S₄, contralat. ulnar nerve stimulated, students' coil (coreless) 10 cm., with rostral region of cerebellum just lateral to spot A, stimulated intercurrently between S₂ and S₃, Berne coil (coreless) 6 cm. Rates of stimulation, 48 and 49 break shocks p.s.

FIG. 2A.—Supraspinatus. At fall of S₁, crossed ulnar N. stimulated, coreless class coil 9 cm., 48 break shocks p.s. At S₂, ipsilateral side of vermis stimulated at point A, coreless class coil 6 cm., 9 break shocks p.s., 4 v., 0.2 amp. (mild to tongue). At S₃, cerebellar stimulation off. Signal time-marker at bottom of record shows incidence of stimuli to cerebellar cortex.

FIG. 2B.—Part of similar record from same preparation on faster plate. The beginning of the cerebellar inhibition is off the plate, and the later stages only are recorded. Cerebellar stimulation, 3 cm. Note the increasing phases of quietude in the string accompanying each further downward step in the myograph. Recovery occurred rapidly after S₃.

FIG. 3A.—Quadriceps, tendon jerks. At fall of S₁ onset of cerebellar stimulus, same as in figs. 1A and 1B. Coreless Berne coil 8 cm., rate 48 break shocks p.s., marked by vibrating key below S₁. End of stimulation at S₁.

FIG. 9.—Lower spring and myograph records of brachialis anticus, upper string and myograph records of triceps brachii (without scapular head). Stimulation of cerebellum between points A and C, 13 break shocks p.s., student's coil coreless. Each break shock falls at the moment when the crochet of the lower vibrating key begins its movement of descent. No change in myograph tension, but progressive increase in string vibrations after each quiet period.

PLATE 34.

FIG. 3B.—Quadriceps. The first jerk on the plate is the fourteenth of a series elicited during continued stimulation of spot B of the rostral surface of cerebellum, Berne coil 6 cm. coreless, 48 break shocks p.s. At fall of S₁, cerebellar stimulation ceases.

FIG. 4.—Brachialis anticus. At S₁, onset of cerebellar stimulation in rostral region, spot A; at S₂ off. Coreless Berne coil, 3 cm., 48 break shocks p.s.

FIG. 6.—Semitendinosus. S₁, stimulation of contralat. ext. popliteal nerve, coreless class coil 9 cm. S₂, point A cerebellum stimulated, coreless, Berne 3 cm. S₃, cerebellum stimulated off. S₄, ext. pop. nerve off.

FIG. 13.—Vastus int. and Crureus. A mild crossed extension reflex had been elicited 1 second before plate was exposed. A single break falls on cerebellar cortex at same point in cycle of movement of key as in fig. 9, Plate 33. Each break shock affects the string record but not the myograph tension.

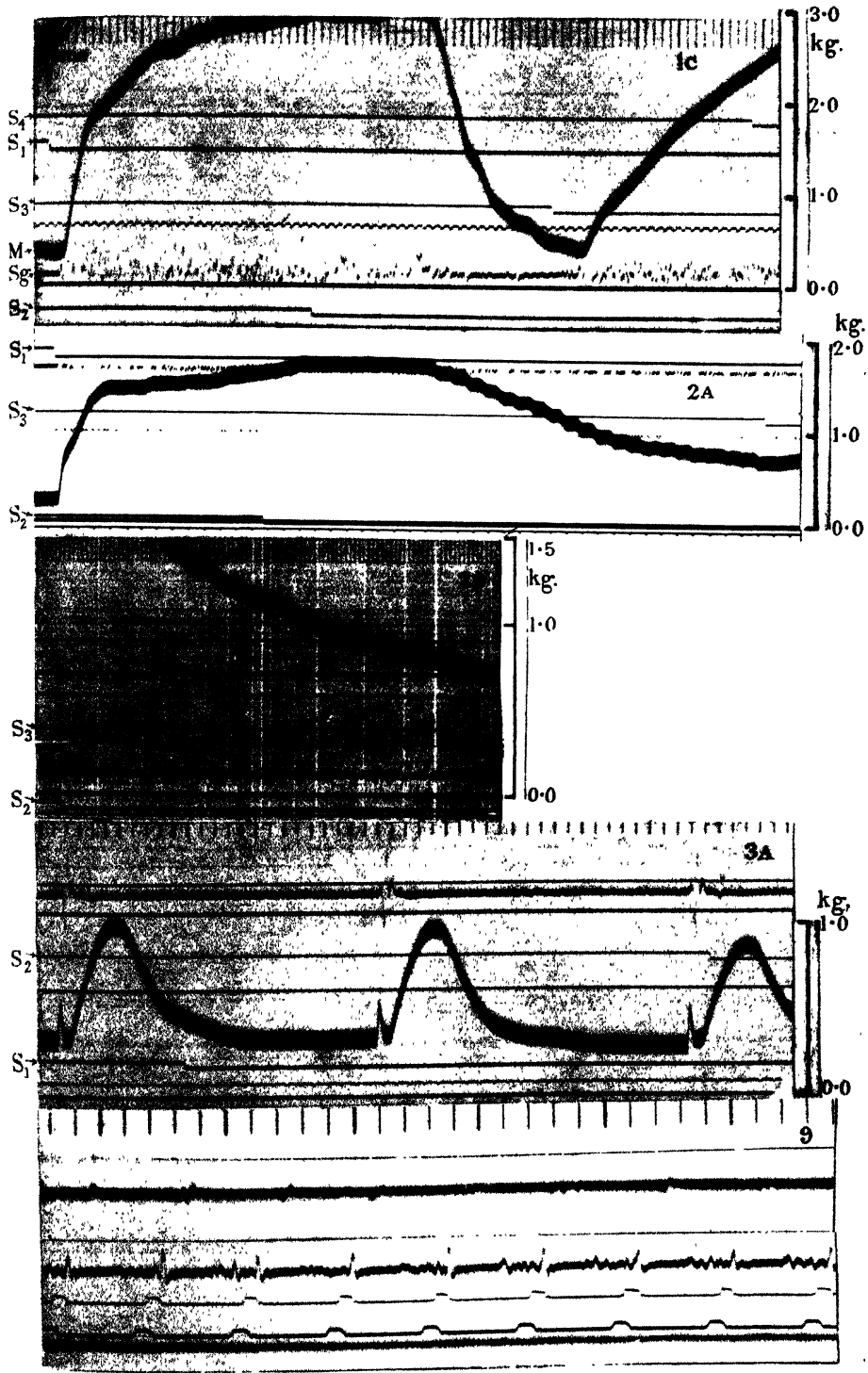
PLATE 35

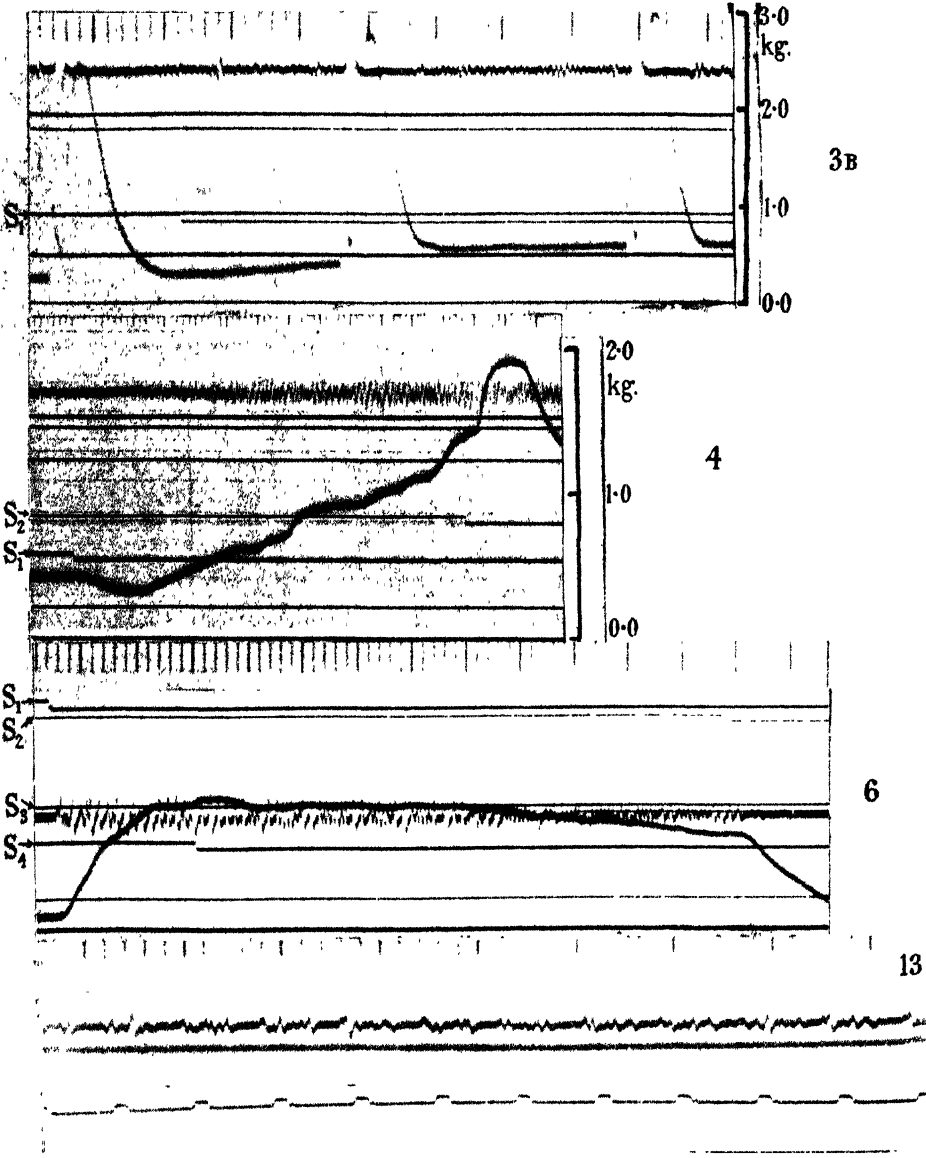
FIG. 12.—Upper myograph triceps brachii without scapular head (note that increase in tension is recorded by downward movement). Lower myograph and string record brachialis anticus. Between S_1 and S_4 , contralateral median nerve stimulated, student's coil, coreless, 11 cm. Between S_2 and S_3 , cerebellum point B stimulated, Berne coreless, 2 cm.

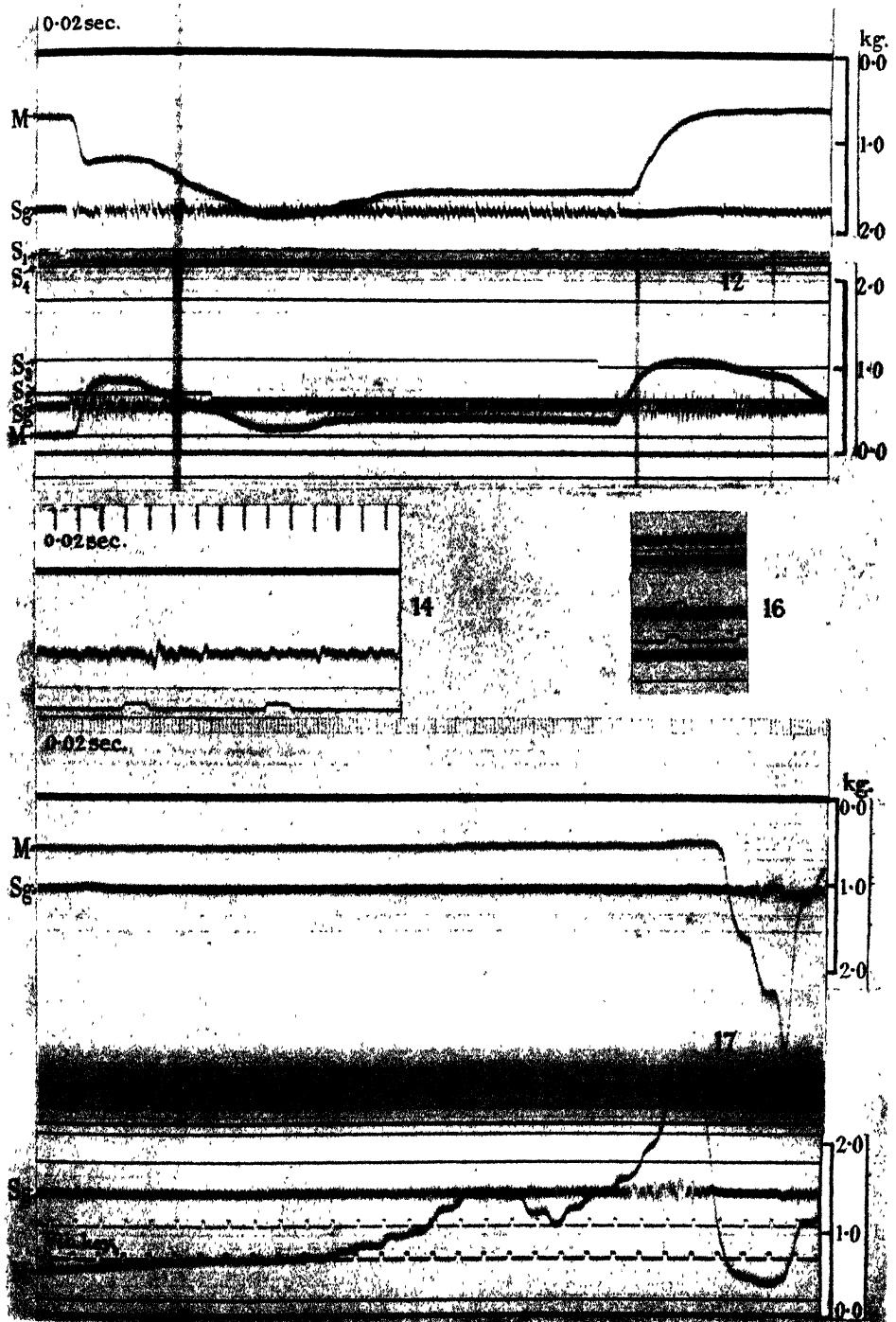
FIG. 14.—String record, triceps without scapular head. Slow series of break shocks, key as before in fig. 9, Plate 33. Berne coreless, 0.0 cm. to point B of cerebellum.

FIG. 16.—Record from same preparation and under same conditions as in fig. 9, taken 3 hours earlier. (9 shocks a second)

FIG. 17.—Upper myograph and string record, triceps (without scapular head). A descent of the upper myograph denotes an increase in tension. Lower record, brachialis anticus. Cerebellum stimulated at point B by student's coil (coreless), 2 cm, break shock p.s. (lower vibrating key, as in fig. 9). After the beginning of the muscular movements, the cortex moved away from the electrodes, so that it was not stimulated for some of the latter part of the record.







Discussion on " Ultra-Microscopic Viruses infecting Animals and Plants."

Sir ERNEST RUTHERFORD, O.M., President, in the Chair.

Sir CHARLES MARTIN, F.R.S.

I am much honoured by the suggestion of the officers that I should open this discussion, and I quite understand that the invitation is extended to me on account of my sympathy with these researches, and not on account of my achievements in them. I am to be followed by a number of gentlemen who have extended the bounds of knowledge in many directions on which I shall touch, and you must look to them to contribute the real value to the debate.

There is one side of the subject on which I have no first-hand information, and that is the side which concerns the virus diseases of plants. That does not matter, however, because I understand that I am to be followed by Prof. Murphy, who will make good all my deficiencies. I think it would have been more appropriate if Prof. Murphy had preceded me, firstly, because plants take precedence in the order of creation, and, secondly, because the first discovery of these filtrable viruses was the work of a botanist and concerned the disease of the tobacco plant. It was in 1892 that it occurred to Iwanowsky (who had been looking for the cause of the mosaic disease of the tobacco plant and had failed to find any visible microbic agency which would account for it) to filter the juice of an infected plant through a porcelain filter. He found that the filtrate was infective for healthy plants in quantities as small as he could handle. The significance of Iwanowsky's observation was unappreciated until seven years later, when the fact was re-discovered by Beijerinck. In the meantime Loeffler and Frosch had discovered that foot-and-mouth disease was not due to any visible microbe but that the contagium was small enough to pass through a porcelain filter. This discovery was the accident of research. Their object was to separate contaminating microbes to see if there was a toxin formed in foot-and-mouth disease.

Following these leads, pathologists investigated whether the infection from a number of diseases of plants and animals for which no adequate cause had been discovered, might not be due to filter-passing organisms, and there are now more than 100 diseases which are attributed to viruses. These include

devastating diseases affecting plants, insects, birds, domestic animals and man. There is nothing peculiar clinically or epidemiologically to this group of diseases. The transport of infection is in some cases direct; in other cases through the air, or by food, or by the bites of insects. It is, indeed, a heterogeneous collection of contagia. The only passport required for inclusion in the group is that someone succeeded in obtaining an infective filtrate, by using a filter which was designed to keep back ordinary microbes, and that no approved microbial causation for the disease exists. The group is continually being added to, and various members are from time to time expelled from it, on the ground that a microbial origin has been satisfactorily established.

There is, however, a peculiarity about some of these virus diseases, namely, the formation of curious bodies in the nuclei or cytoplasm of the cells affected. Many observers have maintained that they represent a stage in the parasite causing the disease. Anyhow, they may be so constant in occurrence and characteristic in appearance that their presence can be used for diagnosis. Their significance is still *sub judice*, but I shall not dwell on this aspect, because we have present Dr. Ludford, who has made a special study of these cell inclusions.

What Dimensions have Filter-passing Viruses?

Porcelain filters were designed by Pasteur and Chamberland to keep back ordinary bacteria. Various grades of clays were experimented with until this was attained. Filters are now made of varying porosity. The interstices of filters are irregular in section and the path tortuous. Whether particles can traverse depends not only on their size, but on the thickness of the filter, the charge on the particles, and whether they adhere to the walls in virtue of surface action. Rigidity and motility also have an influence. Attempts have been made to arrive at the mean cross dimensions of the passages in filters and the dimension of the larger channels by observations on the rate of flow of water under known pressure (Poiseuille's law), and by determining the pressure required to overcome surface-tension and drive air bubbles through a wet filter (Jurin's law) respectively. Both necessitate assumptions which cannot be justified, but are useful for comparative measurements.

From direct experience with very small microbes it may be said that a virus which traverses one of the finer grade filters is probably less than $0.2\ \mu$ in diameter. A particle of this size is just on the limit of clear vision under the microscope, for resolution depends on $\frac{1}{2}\lambda/n.a.$ which, using white light and the best lenses, is about $0.2\ \mu$. By using ultra-violet light and photography an

image of a particle half this size has been obtained. Mr. Barnard, to whom we are so much indebted for the development of the technique of microphotography by ultra-violet light, and whose beautiful demonstration we have just seen, will, no doubt, develop this side of the subject.

There is a great difference in the readiness with which viruses filter. Some, as bacteriophage, and those of foot-and-mouth and mosaic diseases, filter without serious dilution after the first cubic centimetre; others, as those of distemper, fowl pox and other poxes, are so much retained that it is difficult to demonstrate infectivity of the filtrate. It appears, therefore, that great variation in size exists between different viruses, although, as mentioned above, absorption on the walls of the filter must not be disregarded.

Recently many attempts have been made to determine the size of viruses by filtration through collodion films arranged to have varying porosity, using as a gauge of porosity the molecule of egg albumin or hæmoglobin, gold sols, collargol, and colloidal As_2S_3 . Various observers have recorded that collodion membranes, which were but slightly permeable to albumin or proteins allowed one or other of the viruses of mosaic disease, fowl plague, foot-and-mouth disease, rabies, neurovaccinia, herpes and bacteriophage to pass. If the interpretation which they have placed upon their results be admitted, it would indicate that the diameter of the particles of these viruses was of the same order of magnitude as that of a protein molecule, or 2-3 μ . There are many pitfalls in experiments of this nature, and more recent experiments, which seem to me less open to criticism, indicate a magnitude equal to or greater than that of the particles in the colloidal silver preparation "collargol," viz., 20 μ .

This would equal in size a conglomeration of 500 to 1000 protein molecules, which there is no reason to regard as inadequate to possess the properties of a living organism.

Are Viruses Small Organisms or Ferments?

On account of their small size, and their failure to propagate except in the presence of living plant or animal cells, viruses are regarded by some eminent bacteriologists as more akin to ferments than to organised beings. This view has been ably defended, more particularly in the case of mosaic disease, bacteriophage, and the infective sarcomata of fowls. According to it, the virus is a catalyst which, when it enters the living cell, upsets its metabolism in such a way as to lead, not only to the functional derangement of the cell, but to a vast increase in the catalyst, which, being set free, infects other cells. Such a conception of an inanimate catalyst, reproducible in this way, does, I admit,

explain many facts, but there are others which appear to me inexplicable on this hypothesis.

Reduced to the simplest terms, a living organism is different from inanimate material by its powers of assimilation, multiplication and variation (imperfect assimilation). The evidence seems to me to indicate that viruses display all these properties. I can arrange the present facts more tidily in my mind on the assumption that viruses are simple organisms, which appear to be obligatory parasites, owing to their limited powers of assimilation. Considerable support is given to the view that viruses are obligatory parasites by the apparent absence of saprophytic viruses. Of ordinary microbes only a fraction of 1 per cent. are pathogenic to animals or plants; the others subsist upon inanimate materials—proteins, carbohydrates, and simpler carbonaceous and nitrogenous molecules. If saprophytic viruses occurred they would surely manifest themselves by the products of their activity, as microbes do. Moreover, if they were widespread, we should never be able to purify a pathogenic virus by filtration, because the materials used would always be contaminated.

I do not know of any properties of viruses, other than size and apparent inability to thrive apart from living cells, which distinguish them from visible microbes. Neither their resistance to chemical and physical agencies nor the nature of the immunological response of animals infected by them is essentially different. They are variable, can be modified by passage through different plants or animals and educated to withstand disinfectants.

I should occupy too much time were I to relate the facts in justification of these opinions. The subject should be a fruitful one for discussion, and I must rest content with having formulated them for your criticism.

Prof. PAUL A. MURPHY.

The virus diseases of plants give the impression of being a more homogeneous group than the animal virus diseases appear to an outsider. It may be that, particularly among the latter, some diseases are included which are non-virus in origin, but taken as a whole it has never been doubted that the underlying causes of both groups of diseases belong to a common type.

Virus diseases of the plant kingdom may be artificially divided into two classes—(1) capable of mechanical transmission; and (2) not so transmitted, but capable of being experimentally conveyed by means of grafting or budding. It will be noted that the former class alone can be proved by filtration and inoculation experiments to be caused by a filter-passing virus. Nevertheless, there is no reason to doubt that graft-conveyable diseases are also of virus

origin, and for two reasons: their strong family resemblance to the proved virus diseases, and the fact that, like the latter, they are known (or presumed) to be insect-borne. Indeed, transmission by means of grafts is looked upon as one of the hall-marks of a virus disease in the plant world, because there are so very few truly systemic diseases of plants outside of this group.

In comparing animal and plant viruses, one is impressed more by their similarities than their differences. In the first place, viruses in the plant kingdom, as among animals, are known to occur and increase only in the places in which they produce their specific effect. They have not been found outside of the plant in which they cause disease, or of its remains. There may be some exception to this, notably in the case of a mosaic disease of wheat, which persists for years in the soil; but whether in the remains of a diseased crop or independently appears uncertain. There is, however, no convincing evidence that the agent of virus disease in plants has ever been propagated outside of its natural host; although it is suggestive that, when extracted and diluted sap of diseased plants is allowed to stand for some time it is said to give rise to a greater percentage of successful infections than when used at once.

The systemic character of plant virus disease has been referred to—in marked contrast to almost all other diseases of plants caused by fungi, bacteria and protozoa, which are generally localised. Incidentally there may be some evidence in this that a virus is of an entirely different nature from any of the other agents mentioned. All the organs of the plant are invaded by the virus, with the exception of the seed in most cases. It is natural, therefore, to find that a virus persists from year to year in perennial plants, or in their perennial organs, such as root-stocks, rhizomes, tubers and bulbs.

It was formerly considered the rule that a plant never recovered from a virus disease, but while this is generally the case, a number of recent observations make it possible that recovery may not be entirely out of the question. Some of the cases referred to, however, do not appear to distinguish between apparent and real recovery, for it is characteristic of many plant viruses that certain infected plants (perhaps after an obvious initial reaction) fail to show any symptoms, but are, nevertheless, permanent carriers of infection. Thus in the case of long-standing varieties of potato, such as Up-to-Date, practically every plant is a carrier. The claim has been made that viruses can be originated by inoculating other plants with the juices of these "healthy" potatoes. I have recently been able to show that individual plants of the variety Up-to-Date can be found which are virus-free.

Following the work of Allard and Murphy on tobacco and potato, respectively, it is now recognised that the same plant may be attacked by numerous recognisably, and apparently permanently, different mosaic diseases. The responsible viruses are relatively fixed, and, though they are recognisable only by the symptoms they produce, their objectivity is vouched for by the fact that, on comparison, it has been found that the same potato mosaic diseases have been isolated in Holland and Ireland. Nevertheless, it is possible to attenuate a virus by passage through a slightly reacting plant. How far the virus can be altered in this way is still unknown.

Perhaps the most important difference between plant and animal virus diseases consists in the very great regularity with which insects act as vectors of the viruses of plants. Present knowledge would justify the statement that every virus disease of plants has its appropriate insect vector or vectors, although it is true that in some cases the insect involved is unknown; for the mechanism of transference of certain known infectious diseases of the peach, for instance, has not yet been discovered. In perhaps the majority of cases several insects act as vectors of a disease, while in others only one insect is able to convey a particular virus. In this and other ways, a complex of virus diseases may actually be analysed and separated into its constituents.

Certain mosaic diseases are so infectious that they may be spread mechanically by the touch of hand, or even foot, and are no doubt so carried in many agricultural operations. As a rule, however, virus diseases do not spread by contact, although very infectious; and they are in nature dependent on insects for their spread, and are frequently limited in distribution by the range of the insects which carry them.

The time at my disposal does not allow more than a bare reference to that very important point in common between plant and animal virus diseases, namely, cell inclusions. Incomparably more work has been done in this connection on the animal than the plant side, but later work is tending to show that these bodies are characteristic of a large and increasing number of plant virus diseases. While their nature is still in doubt, opinion among plant pathologists is inclined to view them as products of the host cell.

Dr. J. A. ARKWRIGHT, F.R.S.

The virus of foot-and-mouth disease is a very good example of a filtrable virus because it passes freely through bacterial filters, instead of only with great loss and irregularity as is the case with many so-called filter-passers. If 0.5 c.c. of fluid from a vesicle on a guinea-pig's foot is diluted 50 times with water and

passed through a Chamberland 3, or a Seitz filter, which will remove bacteria, the total number of infective doses contained is often scarcely reduced, or not to less than one-half of the original. After filtration the vesicle fluid diluted 5 million times will often infect a guinea-pig in a dose of 0.05 c.c.

The virus from one susceptible species of animal is often scarcely infective for another until it has been adapted by passage as in the case of many other diseases, bacterial and protozoal. After an attack of the disease an animal is completely resistant for a time. The immunity then passes off gradually, and it is first possible to reinfect by inoculating the places which are naturally the most susceptible, such as the soles of the feet and the tongue in guinea-pigs and the lips and tongue in cattle, that is the sites where vesicles form in a natural attack of the disease. During immunity the blood contains antibodies which can be shown experimentally to neutralise the virus. All the known facts of the immunity can be explained by reference to the production of antibodies. A vaccine made of virus inactivated with formalin will procure considerable immunity in guinea pigs.

There are three different types of foot-and-mouth disease. An attack of one of these does not protect against another. The distinction extends to the antibodies and to vaccines. Prof. Ciuca showed that the three viruses can be distinguished by complement fixation. The existence of types with serological differences recalls the types known in certain bacteria, *e.g.*, pneumococcus, etc. In spite of the resemblance in behaviour of the virus to a bacterium it is well to remember that the assumption that the virus is a living microbe is based on analogy with visible micro-organisms which can be cultivated on artificial media. Culture of the virus *in vitro* has never been satisfactorily demonstrated, but it increases very rapidly in the living body at the sites of vesicles.

The only alternative to the view that the virus is an independently living microbe appears to be that it is a metabolic product of the animal tissues. Successful propagation of the virus on sterilised artificial media would decide the question of the nature of the virus in favour of a living microbe. The suggestion that the virus is really a poisonous substance of animal origin, if true, would be very difficult to prove, unless its origin *de novo* in the animal body could be established, and all the existing evidence is against this happening.

Propagation of the virus in living animal tissues *in vitro* would not really decide whether the virus has independent life.

Mr. J. E. BARNARD, F.R.S.

The work bearing on the virus problem in which I have been engaged consists mainly of efforts to improve microscopical method and the technique of filtration. The latter is being continued and extended by Mr. Elford, but owing to the time limit imposed, I do not propose to refer to it further, but to confine myself to the use of the microscope. The small size, if that is the chief characteristic of a virus, renders it difficult to make a direct observation on any material at present available. It is generally regarded as possible to observe a body under the microscope down to a certain order of size, but unfortunately that limit is by no means a perfectly definite one. As the physical and optical limitations which are imposed by theory are approached, difficulties increase so rapidly and possible errors become so large that observations may be of little value.

With a view to developing a microscopical method that could be applied to virus investigations I have confined my attention almost entirely to the use of light of short wave-length, particularly in the ultra-violet region. Sir Charles Martin has already referred to the possibility that viruses are not only small but have other characteristics difficult to determine, and it does appear likely that such is true. There is some experimental evidence, for instance, suggesting that the difficulty of observing a virus is in part due to the possession of optical properties similar to those of the medium in which it lies. While that may be so over a given range of visual wave-lengths, it is difficult to imagine that it can be true for all available wave-lengths. If, therefore, a body has the same refractive index over the middle part of the visual spectrum, it is highly improbable that it will maintain that similarity in any part or parts of the ultra-violet region. That is at least one reason for continuing work with light of short wave-length, and may partly account for the difficulty of securing visibility.

The wave-length of the light used in microscopic work largely determines the order of resolution that can be achieved. Thus by the extension of the available radiations into the region of the ultra-violet it is possible to resolve, that is to determine the size and form of, bodies smaller than can be seen by visual light. That side of the research has to be considered in relation to the particular biological problem involved, but in general resolution increases with frequency. On the physical side perfection of optical apparatus for use with any radiations transmitted by quartz presents no theoretical difficulty, but there are practical problems to be overcome which may take time. It

does not appear desirable, however, to devise appliances which would be usable with an ideal object but which would at present be quite useless in attacking such a biological problem as the viruses present. Apparatus is now in use with which bodies can be photographed smaller than it is possible to observe with an ordinary microscope, the operations being certain and accurate.

There are two directions in which progress is essential. One is to obtain greater visibility, and this is in process of achievement by a dark-ground ultra-violet method of illumination. The other is to devise methods of illumination by which short exposures, down to one-tenth of a second, can be made. One of the difficulties of ultra-violet light work, and one which appears likely to persist, is that the source of light must be of the nature of a spark. It will probably be better to maintain that type of illuminant, as it emits light which consists of lines or small groups of lines with narrow ranges of wavelengths, but it follows that the amount of available energy is not large. The slides shown indicate the direction in which research is proceeding. Thus the morphology of the larger bacteria is clearly shown, whereas some structure is to be observed, even in the smallest.

Dr. KENNETH M. SMITH.

In the great majority of plant virus diseases, the infective principle is spread from plant to plant by the agency of insects, and in considering this relationship between virus and insect attention is drawn to the following points. Firstly, is the insect an obligate alternate host for the virus, or merely a mechanical carrier? In the mosaic group of viruses where direct inoculation by the needle is often possible, it is reasonable to suppose that no obligate connection with the insect exists, and in such cases the insect is presumably a mechanical vector. In certain other plant viruses, however, the insect does not become infective until a period of approximately 14 days has elapsed from the first feeding on the diseased plant. Here then, unless we explain this period as the time taken for the virus to pass into the body of the insect and return via the salivary juices, is a case where there is some obligate connection between the virus and its insect carrier. Experiment has shown that plant viruses can remain infective in the body of the insect carrier for periods ranging from 7 days to 2 months without the insect again having access to a diseased plant. Again, we have found that potato leaf-roll is not inheritable by the offspring of infected insects, nor is there any evidence to show that insects infected with plant viruses are in any way diseased or present any abnormal appearance.

Some new points concerning the behaviour of a plant virus and its insect vector are presented very shortly. The virus of potato mosaic when inoculated by means of the needle into a tobacco plant produces an infectious virus disease known as "ringspot," in which the chief symptom is the formation of double concentric rings with a central spot. When ringspot is returned to healthy potato by the needle, a mosaic disease is reproduced, resembling the original disease in some points but differing in the more brilliant mottling and the enormously increased infective power. When inoculation is made from the original mosaic potato to tobacco by the aphid instead of the needle, a different disease is produced, the chief symptom of which is the formation of dark green lines following the veins, accompanied by mottling. Again, it has been found that continuous needle inoculation of ringspot through succeeding generations of tobacco plants entirely alters the character of the virus. During this progressive series, the symptoms pass through rings, then lesions and finally rapid death of the plant. That this is an actual increase in virulence, and not one lethal to the tobacco alone, is shown by the effect of its needle inoculation into potato where it produces the intensified mosaic typical of ordinary ringspot inoculation with the additional factor of death. Now using a tobacco plant affected with this virulent disease as the source of infection, it is possible to produce two different diseases in healthy tobacco plants—one by needle, one by aphid—or to infect the same plant with both diseases by using these two methods of inoculation.

By certain manipulation of the virus of potato mosaic, it is therefore possible to change its character and, by starting with the normal mild disease upon potato, to produce from it four apparently distinct diseases, or perhaps it would be more accurate to say, four distinct modifications of the virus, *i.e.*, (1) ringspot of tobacco; (2) its highly infectious counterpart in potato; (3) the virulent form of disease in tobacco; (4) the aphid-produced green line disease, also in tobacco.

Dr. W. E. GYE.

The filtrable tumours of fowls were discovered by Peyton Rous at the Rockefeller Institute in New York. They constitute an important group of new growths because an infective filter-passing agent can be extracted from them easily. In this respect they stand in sharp contrast to the tumours of mammals. The agent may be obtained in many ways, but we have found that the best method of obtaining it in most active form is to break up the tumour tissue with sand, and extract with a saturated salt solution, which is then slowly

diluted with distilled water to physiological saline strength. The purpose of the saturated salt solution is to break up and dissolve the nuclei of the cells with which the agent is apparently associated. The filtrate thus obtained is usually infective in doses of about 0.001 c.c., and the tumour produced is a true tumour, developed from the connective tissues of the animal which is inoculated. From this tumour further infective filtrates may be obtained.

Thus the essential phenomenon of filtrable virus diseases is met with here, namely, that apparently the agent can reproduce itself indefinitely in the infected tissues of the animal. Therefore, the arguments which are put forward to back the belief that, for example, the virus of dog distemper is an organism, apply equally to tumours which filter in this way. But this argument is not accepted in regard to fowl tumours. This is not mere caprice; there are reasons for it. In the first place, it must be noted that there is a whole group of filtrable tumours of very diverse structure—simple cell tumours, complex tissue tumours, tumours containing cartilage and bone, others having pigmented cells, and one which consists of pure capillary endothelium. These tumours differ from one another as much as a cat differs from a dog, in structure, biological characteristics, and so on; but they all exhibit the one common fundamental property of new growths: unlimited cell-proliferation. Each one of these tumours yields a filtrate which will infect fowls only. In fact, the filtrate has the properties of the cells from which it is obtained, in that it is specific to fowls, and gives rise only to tumours of the type from which the given filtrate is obtained. If, for example, a fowl be inoculated in one breast with filtrate obtained from the simple spindle-cell sarcoma, and in the other breast with filtrate of endothelioma, in the one breast simple spindle-cell sarcoma will result, and in the other breast an endothelioma. So that if we accept the view that the causative agent in these cases is a filtrable virus, we are faced with a very difficult problem and one which has no analogy in bacteriology.

There are two aspects of the problem: the one purely bacteriological, the second pathological, which is concerned with the nature of new growths. This group of tumours conflict with widely believed classical doctrines. It is the common opinion, held for a half a century, that the cause of tumours is biological—a morphological variation, or a physiological perversion of cells—and that there is no extrinsic agent taking part in the formation of tumours. When Rous first described these filtrable tumours most investigators refused to accept them as being true tumours. To-day, after 18 years, they are universally accepted. Then, what is this ultra-microscopic causative agent? Some pathologists believe that the agent is an enzyme, although the agent has

all the properties associated with a virus, but none of the properties of enzymes. In France, Prof. Roussy rejects both virus and enzyme. He says that the agent is a " physico-chemical principle "—I do not know what that means.

What are the elementary facts in connection with the filtrates? In the first place, by heating a filtrate up to a temperature of 55° for 15 minutes the filtrate is inactivated. If it is heated at a rather lower temperature or for a shorter time, the agent is attenuated. The activity of a filtrate diminishes rapidly at body temperature. This is a property which is known for a good many filtrable virus diseases, and it is known, in fact, for larger organisms. The loss of potency due to incubation is caused by the activity of tissue oxidases and can be inhibited by means of HCN. A filtrate retains its activity for 2 or 3 days at 37° C. in the presence of 1 : 20000 HCN. This phenomenon has at least one parallel in general bacteriology, viz., in the fact that the autolysis of meningococci is prevented in a similar way (Flexner).

The next observation I should like to make is on the question of antiseptics. The agent of these tumours is susceptible to the action of antiseptics, and to the whole range of antiseptics as we know them, and as we apply them to ordinary bacteria. One particular antiseptic which I have studied very carefully indeed is acriflavine. Acriflavine will act in the presence of serum, but the state of the serum is important. When the serum has been inactivated by heat (or by age) the concentration of the acriflavine required to kill a micro-organism is much higher than when the serum is fresh. With the virus of bovine pleuropneumonia, for example, the concentration is 1 : 5000 with old or heated serum, 1 : 40000 with fresh serum. The viricidal powers of serum summate with the antiseptic powers of acriflavine. This phenomenon occurs when filtrates of tumours are treated with acriflavine and serum.

From this and other evidence I believe that the agent of these tumours is a virus, and that the virus is of the same order of a living thing as ordinary microbes.

(The Discussion was adjourned until March 14.)

Prof. J. C. G. LEDINGHAM, F.R.S.

I propose to deal briefly with certain contributions which the study of vaccinia virus has made to our general knowledge of viruses and their interactions with the tissues of the host.

(1) In view of the importance of specific immunisation methods against

virus disease, it is not irrelevant to recall the transformation of variola virus to vaccinia by passage through lower animals—a transformation which was the means of securing a fairly lasting immunity against risk of, and death from, smallpox. So far as animal viruses are concerned, it is doubtful if the possibilities of securing attenuated living variants for immunisation purposes have been adequately explored. The precise duration and degree of protection afforded by killed viruses in experimental foot-and-mouth disease, distemper, etc., are as yet uncertain. So far as vaccinia is concerned, results with killed virus have shown that little immunity is conferred. As has been shown for bacteria, however, the antigenic constitution of the variant, used in the killed state for immunisation, may be all-important, and this point has not yet been investigated in the case of viruses.

(2) The special affinity of vaccinia and certain other viruses for epiblastic tissue, as postulated by some workers, has not been generally accepted. The available evidence points to a primary implication of reticulo-endothelial elements. In this connection the importance of the condition of the seed-bed into which the virus is introduced, is now recognised. As with local bacterial infections, it has been shown that the proliferation of virus may be inhibited or annulled when introduced into skin areas which have been the sites of some antecedent non-specific inflammatory reaction. With regard to the disputed question of the mode of action of antiviral serum I have noted that dermal sites, in which the introduced virus has been incompletely neutralised by the serum quota, show little redness and œdema, and histological examination reveals a strict localisation of the tissue response. It is possible that just as one observes a localisation of the tissue response to diphtheria bacilli when introduced with antitoxin, antiviral serum may sensitise the virus for phagocytosis. An antitoxic effect is also conceivable.

(3) The elementary bodies in vaccine lymph which were described by Paschen in 1906 seem to me, from recent studies I have made of them, to deserve more serious attention, as possibly representing the actual virus. In the papular stage of the vaccinia lesion these bodies increase enormously in numbers, remaining however quite free. With vesicle formation the bodies tend to congregate round cells, and indications are found of phagocytosis of these bodies by the cells of the exudate. Whatever their nature be finally proved to be, their behaviour in the lesion recalls that of a staphylococcus or streptococcus after introduction into the dermis of a susceptible host.

(4) My last point concerns the activation of latent or carrier bacterial infection by virus action, of which the classical example is the activation of

B. suispestifer by the swine-fever virus. Recently it has been claimed that intravenous inoculation of rabbits with a highly virulent strain of lymph results in the development of macroscopic necrotic lesions in skin and internal viscera, which have been interpreted as examples of generalised vaccinia. I have also during the winter months encountered such lesions under precisely similar conditions, but would interpret the phenomena quite differently, in view of the development in animals so inoculated of acute catarrhal eye and nose affections. From these catarrhal lesions and from similar lesions in unvaccinated animals, organisms, belonging mainly to the *Pasteurella* group, have usually been readily recovered. The histology of the visceral and skin lesions is that of uncomplicated *Pasteurella* infection, which may attack both skin and internal organs.

I take the view, therefore, that these gross lesions of skin and organs described in rabbits after intravenous inoculation of neurovaccine, cannot be certainly labelled as generalised vaccinia, unless carrier *Pasteurella* or similar infections, capable of being activated by vaccinia, are definitely excluded. The interaction between viruses, or between a virus and a latent bacterial infection, in the same host, is likely to become a most important subject of study.

Dr. R. N. SALAMAN.

I will confine my remarks to general principles affecting the potato, which may be deduced from the work we have been doing in Cambridge. The first is contained in the statement that it is possible that disturbances induced by the introduction of foreign protoplasm into a host plant may be the cause of virus disease. Johnson found that when he injected juice from "healthy" potatoes into tobacco, he obtained a virus disease; but that when he used seedling potatoes, he failed. Schultz also obtained evidence of virus disease in a "healthy" potato host plant when he injected the juice of another "healthy" potato plant into it. We have repeated both experiments in Cambridge many times, with negative results. I think the explanation is that we have been experimenting with healthy potatoes, whilst the others were using "carriers." Schultz's potatoes were, in fact, not healthy, but carried a Streak virus; he sent me a series of them and I found that every one was carrying Streak. We may conclude that virus disease of potato or of tobacco is not due to mere physiological disturbance.

The question of tolerance to virus infection in the potato, reduces itself to a consideration of the problem of "carriers." Let us take as an example the virus disease, mosaic in Kerr's Pink, a widely grown variety; it is often difficult

to find one plant in a field which has any visible disease in it. When, however, we take cuttings from such plants and graft them on to healthy test plants, they induce mosaic in them. Close inspection in the field will demonstrate here and there an indication of mosaic disease; Kerr's Pink is a very good carrier of mosaic, whilst such varieties as Arran Victory and President are bad ones.

Another disease, carried by some varieties and not by others, is Streak. Irish Cobbler and Green Mountain carry Streak often without showing any sign of it, whereas Arran Victory and President will immediately succumb to it. It is of interest that our best and most lasting varieties of potatoes, such as Up-to-Date, King Edward, and Champion, are all efficient "carriers."

My next point is that "tolerance" may lead us to distinguish two viruses when both produce identical symptoms. This is rather involved, but I will make it clear. There is a disease in the potato, described by Murphy, and called Crinkle. It produces a bold, coarse mottling, with deformity and necrosis in the leaf. We have found, by grafting certain apparently healthy King Edward plants to Arran Victory and to President, that the Arran Victory goes down with severe Crinkle, and that President remains free from it. On the other hand, we have a Crinkle disease in the Champion variety and many others which, when grafted on to Arran Victory and President, produces Crinkle in both. Further research has shown that we have two viruses: one, Crinkle "A," which produces disease in both Arran Victory and President, and another, Crinkle "B," which has no effect on President, but has on Arran Victory. Time forbids reference to other differentiating reactions. (Dr. Salaman then threw slides on the screen illustrating this point.)

A word as to alteration of virulence. That the virulence of a plant virus can be attenuated has been shown by Kunkel; and that it can be increased by Dr. Kenneth Smith, but in Crinkle "B" which I have just discussed. "passage" through President— a variety tolerant to Crinkle "B"— has not attenuated the virulence of the virus, nor has the "passage" through Arran Victory, which is very susceptible, increased it.

Varietal reaction is of great importance in the potato. The following will show how vital a part it must be allowed in our investigations. Varieties may react differently to the same virus or group of viruses; there is a virus which we call "Interveinal Mosaic," because it causes a pallor between the veins of the leaflet. In President and Arran Victory it produces its characteristic picture. If you put the same virus into Schoolmaster, you find that instead of "Interveinal Mosaic," you get a definite margin of pallor all round the leaf,

which has earned itself a separate name—"Marginal Mosaic." Again, in most varieties, if Leaf Roll and Mosaic occur together you get a result in which each disease is obvious. In Rhoderick Dhu it produces an entirely different picture, which has been described as a new disease, under the name of "Rolled Edge."

(Slides and description.)

The converse is also found; the same clinical picture in different varieties may be produced by different viruses. Thus, "Marginal Mosaic," described above, can be produced by the following viruses in different varieties. In the variety Schoolmaster, it is due to the virus of Intervascular Mosaic; in Catriona, it is due to the virus of ordinary Mosaic; in Great Scot, it is due to a mixture of the viruses of Leaf Roll and Mosaic.

Prof. F. W. Twort.

I purpose to note certain facts, to relate some observations and to suggest a line for future research. First, consider the theories of evolution. If these are accepted, one must admit that bacteria are too highly organised to represent the start of life, and that there must exist forms much more primitive in Nature. If that be so, it seems reasonable to think that viruses may be pathogenic representatives of some of these forms. Prof. Martin has observed that we have no evidence that non-pathogenic varieties exist: but as pathogenicity is the only manifestation of a virus, it is not surprising that we cannot demonstrate non-pathogenic varieties. Judging, however, from what we know of ordinary bacteria, they should exist. Moreover, we cannot say that our "pure" bacterial cultures are not contaminated by such forms. The bacteriolytic (bacteriophage) may belong to a group that under certain conditions becomes pathogenic for the bacterium and is detected only when lysis occurs.

Another point is that most of the work carried out on viruses has been by ordinary bacteriological methods. If, however, viruses are representatives of more primitive life, they must have existed before the organic world was evolved, when they could not have had organic material for their foodstuff, and any energy obtained must have come from some other source. May I put forward these observations?

I have noted that with the bacteriophage, the phenomenon of "spontaneous origin" frequently takes place in several tubes at about the same time. Also that the inflammatory conditions called "sore throat" often start about 24 hours before a change is observed in climatic conditions. The rapid souring of milk

during electrical disturbances is well known, as is also the change in plant vitality. The growth of bacteria when placed in a copper-lined incubator may differ from that obtained when they are grown in a cupboard at the same temperature.

We know that electro-magnetic vibrations affect living organisms, and that the effect varies with the frequency and with different types of organisms. Certain rays of light and heat are essential to man, while other rays of a much higher frequency are detrimental, and light rays are generally harmful to bacteria. On the other hand very little is known about the effect on different forms of life of rays of a lower frequency; but it is known, and I have often observed, that living tissues collect certain wireless rays to a marked extent. Short waves, modulated by speech or music and transmitted from such a country as America, can be impressed on the body of a man, for, if transferred to suitable apparatus and demodulated, the original sounds are reproduced.

In conclusion, electro-magnetic waves affect living beings. Some have no apparent effect, others may be beneficial or harmful according to the frequency of the wave and the type of life. I suggest that viruses may be affected also, though the frequencies of active rays may not always agree. My experimental results were not entirely negative, but lack of funds and necessary apparatus stopped my researches.

Dr. C. H. ANDREWES.

There are certain general differences, from the point of view of immunity, between bacteria and viruses. Firstly, most virus infections of animals leave behind them an immunity which is solid or even absolute--absolute in that it cannot be broken down, as can immunity against most bacteria, by giving sufficiently large doses of the infecting agent.

Secondly, the immunity is usually very lasting, or even life-long. Such a lasting immunity is possibly associated with a latent persistence of living virus somewhere or other in the immune animal; this is certainly so with the salivary virus of guinea-pigs.

Thirdly, it is not as a rule possible to immunise or to evoke the production of antibodies by means of heat-killed viruses. It is, however, often possible to immunise by inoculation of viruses inactivated by phenol or formalin. Most workers are convinced that such vaccines are actually dead; but others hold that this is unproved. It is hard to understand why killed viruses should

evoke the appearance of antibodies *only* when inoculated into a species which is naturally susceptible to the living virus.

Fourthly, the antibodies found in the serum of animals which have recovered from a virus infection seem to be rather different from other known antibodies. If immune serum is mixed with the appropriate virus and injected into an animal, one does not see the local or general disease which would have been expected, had virus alone been given. It has naturally been assumed that an antibody has reacted with, neutralised, or killed the virus *in vitro*; hence the terms "viricidal" or "virulicidal" antibody. Yet it has been found with many viruses that, when serum and virus are mixed and straight-way inoculated into an animal, neutralisation is every bit as good as when incubation of a mixture of the two has been carried out beforehand.

Dr. Charles Todd and I, as a result of some experiments carried out during the last two years, have had our faith still further shaken in the orthodox view of an *in vitro* union of virus and antibody. The following experiment has been performed many times with vaccinia virus:—Some immune serum is mixed with excess of virus—far more than it can neutralise. After contact, say for 24 hours at room temperature, the mixture is passed through an L2 Chamberland candle, which is known to hold back the virus and to let through the antibody; the filtrate is then found to contain as much antibody as it did before. There is no evidence of using up of the antibody by contact with the virus *in vitro*. Conversely, virus can be recovered from a mixture with excess of serum, in which the virus appears to have been quite inactivated. Todd found that an innocuous mixture of fowl-plague virus and immune serum could be rendered lethal to a fowl by simply diluting it with 9 volumes of saline. And dilution will similarly re-activate vaccinia virus in a mixture with excess of serum, even when the two have been in contact for 24 hours. We hesitate to refer to the phenomenon as a dissociation by dilution, such as is familiar to workers with toxins and antitoxins, since evidence is at present lacking that the virus and antibody have ever been united outside the body.

Since experiments at present in progress with vaccinia have failed to reveal any evidence of an antigen-antibody union taking place in the blood-stream, one looks at the cell as a possible site for the essential protective action of the immune serum. And yet other experiments, again with vaccinia, suggest that once a virus has entered the cell, the presence of antibody in the fluids bathing the cell is powerless to prevent it from flourishing there. Does the serum possibly act at the surface of the cell, and prevent the virus from

infecting the cell? We are far from getting an answer; but I feel that the facts already known suggest that the body's defence against minute intracellular parasites, such as viruses presumably are, may be rather different from its mode of protection against visible bacteria.

Capt. S. R. DOUGLAS, F.R.S.

1. *Observations on the Migration of Viruses in an Electric Field.*—Before 1927 most of the work to elucidate this problem had been carried out by indirect methods, and the results had been very contradictory. In 1927 Charles Todd devised an apparatus which he has demonstrated to you. Employing this apparatus he showed that the bacteriophage, acting on *B. dysenteriae* (Shiga), always migrated towards the anode. In these and also in the other experiments mentioned below the E.M.F. used was 200 volts. Todd employed a current of $1\frac{1}{2}$ to $6\frac{1}{2}$ milliamperes acting for 1 to 5 hours. The range of hydrogen-ion concentration of fluid containing the phage varied between $pH = 3.6$ to 7.6 , and even in the most acid solution the migration of the phage was still towards the anode.

Olitski (1927) published experiments in which he stated that the virus of foot-and-mouth disease travelled towards the kathode.

In 1928 Martin and his colleagues investigated the electrical behaviour of the virus of foot-and-mouth disease, using Todd's apparatus, and came to the conclusion that this virus always travelled towards the anode, over the limited range of hydrogen-ion concentration he was able to employ on account of the extreme sensitivity of this virus to the action of acid and alkali.

In the same year (1928) Wilson Smith and myself investigated the behaviour of the virus of vaccinia, and here a preliminary difficulty had to be overcome in order to procure virus free from gross particles and excess of albuminous substances. This was accomplished by allowing the virus to diffuse out into Ringer's solution, or broth from portions of rabbit's testicle which had been injected some days previously with neurovaccine. Such diffusates proved highly infective and were usually coloured with altered blood pigments, but did not contain an excessive amount of tissue proteins.

Employing a current of 4 to 10 milliamperes, acting from 1 to 4 hours over a range of hydrogen-ion concentration from $pH = 5.5$ to 8.4 , the virus always travelled towards the anode. It was also found that the tissue proteins present in the solution travelled towards the anode when the hydrogen-ion concentration was on the alkaline side of $pH = 6.8$, but when on the acid side of $pH = 6.8$ the migration was reversed, and these proteins then travelled

towards the kathode. As regards the altered blood pigments suspended in the fluid, these travelled towards the kathode over the whole range of hydrogen-ion concentration employed.

The reversal of the direction of migration of the tissue proteins at a certain hydrogen-ion concentration, and the fact that the suspended pigment always travelled in the opposite direction to the virus, might possibly account for some of the surprising results which have been recorded in the past in certain filtration experiments, such as statements that viruses have passed through filters which held back hæmoglobin or other proteid fractions.

2. *The Distribution of Viruses in the Blood.*—In a number of diseases due to viruses (*e.g.*, cattle plague, fowl plague, vaccinia, etc.) the blood is infective in certain stages of the disease. Various researches have been carried out to ascertain whether the virus was free in the plasma or was in some way connected with the formed elements of the blood. Working on cattle plague, Kolle, 1899, found that when defibrinated blood was centrifuged at 2000 to 3000 revolutions per minute for 20 to 30 minutes, all the virus was contained in the blood cell layer. Nicolle and his collaborators suggested that the virus in cattle plague might be carried by the leucocytes, and in 1912 he showed that this was the case in typhus fever.

Todd and White (1914) during investigations on cattle plague, having repeated and confirmed Kolle's observations, showed by differential centrifuging that the upper layers of the blood cells, in which the majority of the leucocytes were situated, contained at least 100 times as much virus as the deeper layers. Todd (1928) by a similar method showed that in the case of fowl plague the white leucocytes were at least 100 times more virulent than either the red cells or the plasma.

Wilson Smith (1929), working with vaccinia in my laboratory, found that even after the intravenous injection of the virus the plasma was infective for only 24 hours, but the washed blood cells were infective up to the eighth or ninth day. When the white cells were separated from the red no virus could be demonstrated in the latter, while the former gave evidence of a large amount of virus. He also showed that while the leucocytes contained active virus up to the eighth or ninth day, the plasma after the second day contained antibodies against the virus. This observation would explain the very irregular results which have been obtained by various workers who have employed skin scarification or intradermal inoculation to demonstrate the virus in the circulating blood.

Dr. EDWARD HINDLE.

There are only one or two points, bearing on yellow fever virus, to which I should like to refer. Yellow fever virus is only moderately filtrable; it passes through the coarser filters but is arrested by the finer ones; but one very peculiar fact emerges from filtration experiments, namely, that the virus has a different size in the blood from what it has in the mosquito which transmits the infection. This suggests that some form of evolution takes place in the intermediate host. The virus in the mosquito is arrested by even the coarsest kind of filter; and I should very much like to know if Dr. Kenneth Smith has made any observations on this phenomenon in the aphid which transmits the mosaic disease.

It may be argued that yellow fever is not due to a filtrable virus, but it falls into line with most of the others. You get the same eosinophile inclusions and also it is filtrable, which after all is the main feature of virus diseases. Another point which I think supports the view that the virus of yellow fever is a living organism is the undoubted variation in the mortality of epidemics, in which you get variations ranging from a mortality of 10 per cent. up to one of 80 or 90 per cent. I think this can only be explained by assuming that the virus is showing the same variation which you get in all ordinary plants and animals.

I should like to associate myself with the remarks made by Dr. Andrewes. The features of immunity in most of these animal virus diseases do seem to be distinct from what we know about immunity in bacterial diseases. The very solid immunity is a peculiar property; and with regard to it being associated with tissues, yellow fever gives a rather curious example of that. I have noted one or two experiments on this point. I perfused monkeys convalescent from yellow fever, in order to get rid of immune serum, and inoculated pieces of their tissues into non-immune monkeys, in which I was trying to reproduce the virus. Nothing resulted. But when I subsequently inoculated these monkeys with yellow fever virus it was found that those which had been inoculated with pieces of liver and spleen were immunised against the subsequent dose of virus, whilst those which were inoculated with kidney tissue or brain or lymphatic gland were still susceptible to the virus. That suggests some form of immunity associated with particular tissues. I inoculated the virus in one case a fortnight, and in the other case a month, after inoculation of the tissue.

There is one other point, and that is the duration of immunity. In the case

of yellow fever, the inoculation of presumably killed virus produces immunity in monkeys. The duration of this protection has been tested up to 5½ months and shows no sign of diminution.

Dr. W. B. BRIERLEY.

There are one or two things I would like to say as the result of having listened to the discussion, and of having been in close association with Dr. Henderson Smith who is carrying out virus disease investigations at Rothamsted.

An outstanding difference, to my mind, in the study of animal and plant viruses is the value which is given to the character of filtrability. The plant pathologist does not attach anything like as much importance to this character as the animal pathologist, and is rather inclined to think that a good deal of nonsense has been written about it. Further, certain of the plant viruses are not filtrable at all, or, at least, if they are, we know nothing whatever about their filtrability, because the virus cannot yet be obtained in plant juice. The only way of transmitting these particular diseases artificially is by grafting or budding, or by the use of appropriate insect vectors—apparently they are transmitted in nature only by insects—so that the filtrability of these viruses cannot be tested. The character of filtrability, therefore, which has sometimes been regarded as the portal of entry to the group of virus diseases, does not apply at all in these cases which yet, undoubtedly, are virus diseases.

A noticeable feature of plant viruses is their existence as more or less closely related strains which produce characteristic symptoms in the host and give the appearance of being distinct entities. These strains are capable, under artificial conditions, of being attenuated or of having their virulence increased, but of a possible relationship between strains derived by modification of an original parent and the strains existing in Nature, we know nothing.

A striking feature of plant viruses and one I would like to emphasise, for I do not think quite enough has been made of it in the discussion, is the specificity of insect transmission in certain diseases. Generally speaking, one insect may transmit more than one virus or one virus may be transmitted by more than one insect, but, in certain cases, there appears to be a strict adaptation of a particular insect to a particular virus, and, in at least one instance, that of Aster Yellows, there is a good evidence of a definite "incubation" period in the vector. An interesting case, a little difficult to understand, has just been published by Hoggan in America. Cucumber mosaic and tobacco mosaic, two easily juice-transmissible diseases, can be inoculated into tobacco producing

a combination disease. The peach aphid, feeding upon such a doubly infected plant, picks up the cucumber mosaic virus and leaves behind the tobacco mosaic virus; yet its mouth parts must be bathed with both viruses which, artificially, are easily transmitted by mechanical inoculation with the juice. The importance of transmission by specific vectors is becoming very evident, and its study is likely to throw fundamental light upon the nature of virus.

An aspect of plant virus diseases which is of great importance is the study of the very interesting intracellular bodies, which seem to be characteristic of particular virus-host complexes. These bodies are exceedingly difficult of interpretation, but their relation to the presence of virus cannot be doubted. Incidentally I may state that the systemic nature of plant virus diseases does not distinguish them from fungal or bacterial diseases, which, in certain cases, are systemic.

Surveying generally the phenomena associated with plant virus diseases, we may bear in mind, firstly, the existence of strains of virus whose virulence can be increased or decreased; secondly, the juice transmissible nature of many virus diseases with, on the other hand, the exact adaptation of the virus in certain diseases to a particular vector with an "incubation period"; and, thirdly, the presence of characteristic intracellular bodies. It seems to me difficult, in view of these characteristics, to conceive of virus as a propagating catalyst or, in fact, in any way other than as some form of living organism. I would not like to commit myself to the idea of a minute bacterium, but perhaps to some form of organism of the same order of life as the bacteria.

Prof. A. E. Boycott, F.R.S.

The only point I should like to bring before the meeting really arises from what Dr. Twort has said about the ultimate origin of these things. Several speakers have stressed the point that they are very like ordinary pathogenic bacteria in the way they behave. That is true. But why is it that we say it? I think, in the first place, because they multiply, and anything that multiplies we call alive. We also know that they are easily destroyed by heat and other agents, such as antiseptics, which are destructive of live things. They cause definite diseases, and these diseases can be transferred from one individual to another. These are, of course, strong reasons for saying that these viruses must be analogous to bacteria. But several of the speakers also mentioned that they do not feel quite convinced that they are just the same kind of things as bacteria.

Going right away to the other end of the scale, you have phenomena like

those associated with the substances that promote growth. If you cut your finger, in the ordinary way the cells which have been destroyed break up and autolyse owing to the ferments which are set free, and the substances which arise in this way promote growth, and by so doing restore the same amount of cells as was destroyed. But suppose you make a tissue culture of, say, connective tissue, and get it away from the influence of the other tissues of the body and stimulate it with an extract of tissue, generally embryonic tissues. In this way, by spreading it out enough you can grow a great deal more connective tissue than you started with. And at the end of the time if you kill the growth and autolyse it you will get a great deal more growth-promoting substance. In other words, you have a process of multiplication which is not essentially different from what you get with the virus of foot-and-mouth disease. It is always associated with live cells and with the growth of live cells. But I do not think anyone would say that these growth-promoting substances are alive in any ordinary sense of the word.

If you go to the next stage, you have a thing like the cancer virus. It differs chiefly from those viruses which cause epidemic diseases, by not spreading from one person to another. Cancer is a local disease belonging to an individual and remains restricted to that individual. Starting with a tumour you make an extract of it; you filter it. You put it into a suitable animal and get a large tumour formed, and from that tumour can be extracted a great deal more virus than you started with. The multiplication is clear. But you can do something more, because you can "start from scratch." With foot-and-mouth disease, or distemper, you have to start with foot-and-mouth disease or with distemper. But for a cancer virus, you need not necessarily start with a tumour. You can start with normal tissues, and by artificially irritating them you can get them into such a method of growth that when you extract the resulting tumour you will get a thing which behaves like a virus, and with that you can go on indefinitely multiplying tumours and virus.

It seems to me that we have a whole series of agents which are capable of multiplication, and which cause a variety of effects. As to what any virus is, analogy is all we have to go on. If you like to choose one way, you say it is like a bacillus. If you like to choose the other, you say it is more like a growth promoting substance. What is impossible apparently at the present moment is to find any criterion that we can apply to enable us to distinguish between these possibilities.

The Absorption Spectrum of Vitamin D.

By R. B. BOURDILLON, CATHERINE FISCHMANN, R. G. C. JENKINS,
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[PLATE 36.]

Numerous studies have been made of the absorption spectrum of the products of ultra-violet radiation of ergosterol (1). These studies have shown that such radiation causes a decrease and final disappearance of the characteristic absorption band of ergosterol (maximum $280 m\mu$). Several workers have reported the simultaneous formation of a new absorption band (maximum 240), and have attributed this band to vitamin D. Our own observations, however, have shown (2) that the first effect of ultra-violet radiation on ergosterol solutions is the production of an increased absorption in the same region of the spectrum as the absorption bands of non-radiated ergosterol, and we have suggested that this is due to the formation of a new substance with an absorption maximum at 280, and that this substance is vitamin D (3). It has also been shown that the product having maximum absorption at 240 has no detectable antirachitic activity (4).*

We have compared the antirachitic activity of irradiated ergosterol solutions with their absorption spectra, both before and after removal of unchanged ergosterol, and have obtained evidence pointing to the successive formation of three substances (or mixtures of substances). Of these, the first has maximum absorption at 280, and is, we believe, vitamin D; the second shows maximum absorption at 240, and has no antirachitic action; the third is similarly inactive, and shows no intense absorption. We propose to refer to these substances as A, B, and C, respectively.

* After sending this paper to the press we received an abstract ('Ber. d. ges. Physiol. u. exp. Pharmacol.,' 1929, p. 728) of a paper by Smakula, in 'Nachr. Ges. Wiss. Göttingen Math.-physik. Kl., H.1., 49 (1928). We have been unable to find a copy of this paper in London, but infer from the abstract that the author suggests that the first product of radiation of ergosterol has absorption maxima at 293 and $262 m\mu$ (but not at 280 as we find) and that this product is vitamin D.

Absorption Spectra of Solutions containing Ergosterol and its Products of Radiation.

Fig. 1 shows the changes in absorption during the first stages of ultra-violet radiation of an alcoholic ergosterol solution. The curves shown are those of a 0.1 per cent. solution of ergosterol in absolute alcohol, before radiation and after 10, 20, 45 and 180 minutes' radiation. It is evident that in the early stages a considerable increase occurs in the absorption for wave-lengths between 310 and 250. This absorption reaches a maximum after 20 or 30 minutes, and then falls fairly rapidly, with a concomitant increase in the absorption at wave-lengths 230 to 250, as is shown in the 3 hours' curve. The initial effects of radiation in 0.1 per cent. (or stronger) solution depend largely on the rate of stirring of the liquid. If this is not stirred at all, a layer is formed on the side facing the lamp containing a high proportion of substance A, which is itself destroyed by the radiation, and consequently a smaller yield of A is obtained, and the absorption at 280 does not reach so high a value as when stirring is used.

The effects of radiation also depend on the relative quantities of radiant energy present in different parts of the spectrum of the source employed. In the solutions shown in fig. 1 these conditions were regulated as follows :---

The lamp was a K.B.B. atmospheric pressure quartz-mercury arc, run at 2.5 amp., and 125 volts across the arc. The distance from lamp to the centre of the solution was 18 cm. The solution was 0.1 per cent. ergosterol in alcohol radiated in quantities of 15 c.c., in a circular flat-sided silica cell, 5 cm. in diameter and 1 cm. thick, with the two plane sides optically ground and polished. This cell held about 22 c.c., and was rotated during radiation once per second in the plane of its polished sides, so as to secure a controlled rate of stirring of the contents and constant replacement of the layer of solution next the lamp. This was ensured by the nitrogen which occupied the upper part of the cell.

In most of our work we have stirred the liquid during radiation by a stream of nitrogen (previously passed through alkaline pyrogallol, and then through the solvent used for the solution under radiation in order to avoid loss by evaporation), but owing to the difficulty of regulating this accurately, the rotation method was used for the solutions shown in fig. 1.

At intervals a portion of the solution was removed for spectrophotography, and returned to the cell, which was re-filled with nitrogen before continuing radiation.

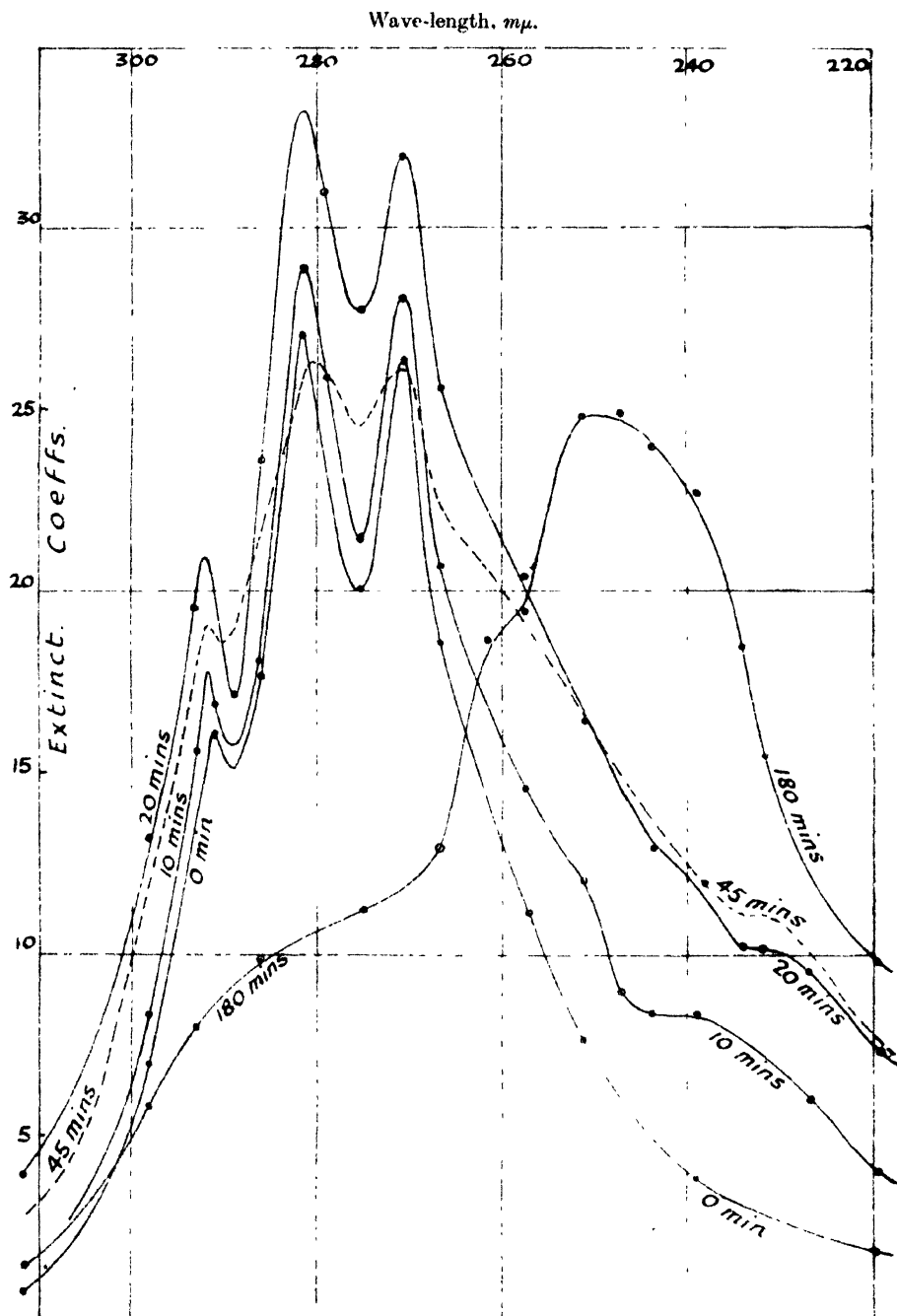


FIG. 1.—Absorption of ergosterol and total products of radiation.
Extinction coefficients in these diagrams are values found for layer 1 cm. thick and concentration of 1 gm. per litre.

Inferences from the Increase in Absorption, and Conditions under which it occurs.

These results are of some interest, since they clearly indicate the formation of a new substance showing intense absorption between 310 and 250 $m\mu$, and itself fairly rapidly destroyed by light. It is not obvious how the initial increase in absorption has escaped the notice of other workers, but we think this may have been due partly to the fact that few workers have measured the absorption during the earliest stages of radiation, and partly to lack of stirring of solutions. We have observed a similar increase in absorption in alcoholic solutions of ergosterol made from yeast by three different manufacturers, and in one sample made from ergot; in solutions in absolute alcohol freshly distilled and condensed in a stream of pure nitrogen, and handled with great care to exclude traces of oxygen; in solutions in ether, in purified hexane (including one sample especially treated to remove possible dissolved oxygen), and in alcohol containing 10 per cent. of water. Variation of concentration from 0.2 to 0.005 per cent. does not appear to affect the increase of absorption seriously, although the time of radiation at which maximum absorption at 280 is observed varies from 3 minutes in the 0.005 per cent. solution to about 30 minutes in 0.1 per cent. solution, with roughly similar rates of stirring. Stirring an alcoholic solution by a stream of oxygen, instead of nitrogen, during radiation, caused no obvious alteration in these early changes in absorption.

On the other hand, the use of a light filter of 5 per cent. alcoholic cobalt chloride solution in a layer 1 cm. thick, which cut off all radiation of wave-length shorter than 265, caused a marked decrease in the formation of substance A, suggesting that the shorter wave-lengths favour the production of A rather than its destruction. With radiation filtered through thick plate glass (*i.e.*, with wave-lengths shorter than 330 excluded) no increase in the absorption of ergosterol could be detected.

Preparation of Substance A in a Concentrated Form, Free from Ergosterol.

Numerous preparations have been studied in which 0.1 per cent. solutions of ergosterol had been radiated, with thorough stirring by nitrogen, for short periods only (30 seconds to 10 minutes), in order to form as little as possible of substances B and C. The unchanged ergosterol was then removed from these solutions by precipitation with digitonin, evaporation of the filtrate to dryness *in vacuo*, and extraction of the residue with ether (which dissolves products A, B and C, but not ergosterol-digitonide or digitonin), as described in a previous paper (5). Throughout the process care was taken to avoid oxidation as far as possible.

Absorption Spectrum of Substance A.

Preparations made in this way show an intense absorption, similar to, but considerably greater than that of ergosterol (for equal concentrations by weight). The absorption curve for solutions radiated only 10 minutes or less, is nearly constant in shape, and is shown by the upper curve in fig. 2, in comparison with the lower absorption curve of ergosterol. It is believed that this curve is an approximate (but not exact) picture of the absorption of a single substance, for the following reasons :—

1. Repetition of the process gives closely similar spectra whether the initial radiation is carried out with an alcoholic or with a hexane solution, and whether its duration is 1 minute or 10. The shape of these spectra only changes seriously (as shown in curve F 17 f, fig. 4) when the period of radiation is increased so as to form considerable amounts of substance B, of which the presence is then clearly indicated by a projection in the curve near 240.

The absolute magnitudes of the absorption per gram of preparation are fairly constant, ranging from 29 to 32.5 units, for preparations prepared in a similar manner after 10 minutes' radiation. Such variation as occurs is probably due to varying admixture of impurities of low specific absorption. Such impurities are to be expected in view of the fact that the preparations handled are very small weights (10 to 50 mg. as a rule) representing all the soluble residues from relatively large volumes of liquid. While careful control tests showed that the process for the removal of ergosterol introduced only negligible quantities of impurity when carried out on non-radiated solutions, it is not probable that we succeeded in avoiding such impurities entirely. Further, the resinous nature of the product A made it very difficult to ensure the removal of the last traces of solvent or of water, and this alone would cause variation in total absorption. It is also possible that oxidation may not have been wholly prevented, in spite of the use of nitrogen at various stages of the process. This would cause contamination with products of low specific absorption.

2. Of substances showing marked ultra-violet absorption, ergosterol and substance B are the two most likely to be present as impurities in these preparations of A. The absence of large quantities of B is shown conclusively by the small absorption at 240. The absence of ergosterol is more difficult to prove, but the following evidence shows that only small quantities (if any) were present : —

- (a) Preparations of substance A were soluble in an equal weight, or less, of alcohol and other organic solvents, whereas ergosterol only dissolves to about 0.3 per cent. in alcohol at room temperature.

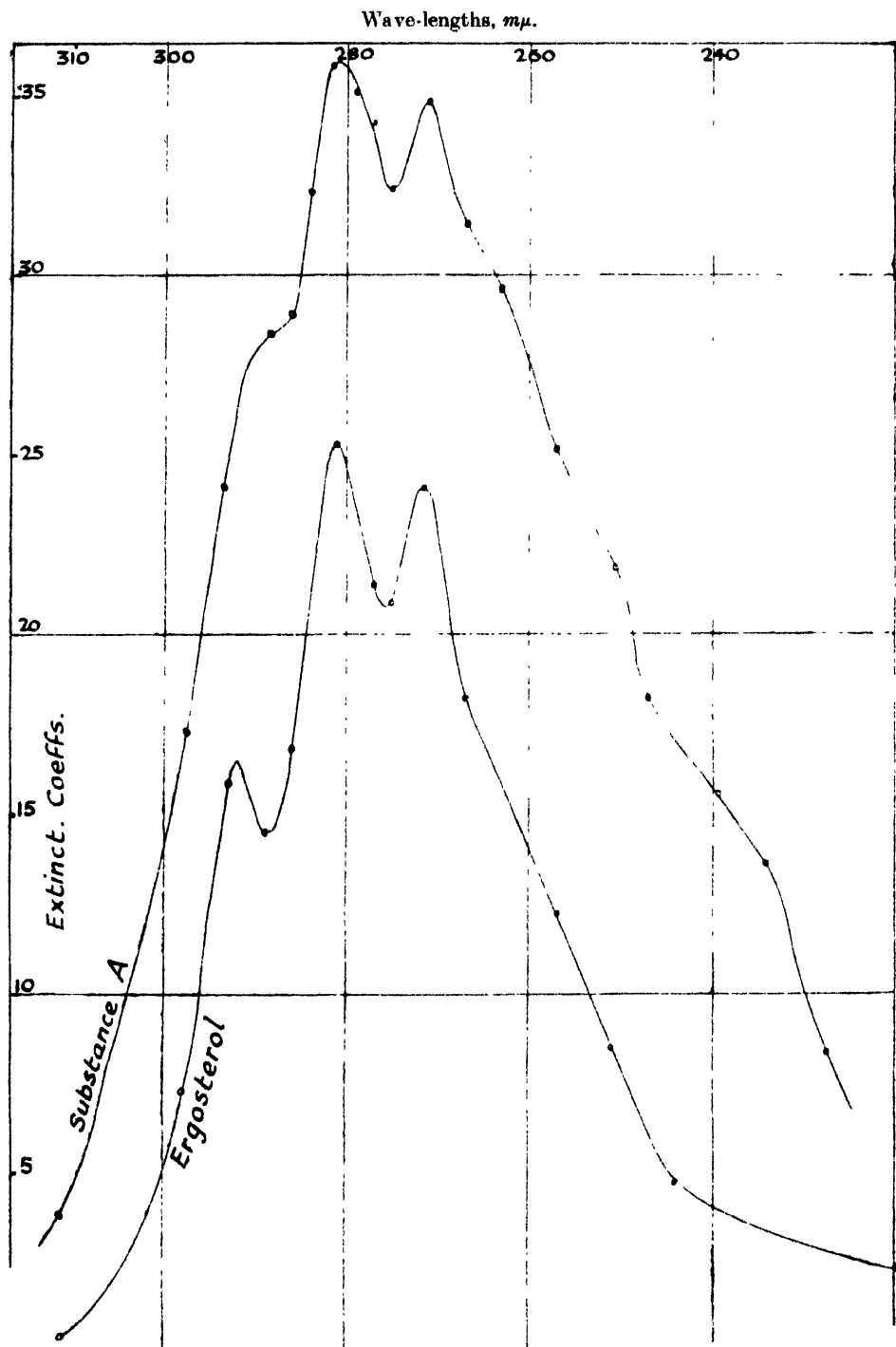


FIG. 2.—Absorption of Substance A (Vitamin D) and Ergosterol.

- (b) Solutions of A (0.1 per cent. in alcohol) gave no precipitate with digitonin, but if only 0.002 per cent. of ergosterol was added, a precipitate was then formed with digitonin, and if larger proportions were added the bulk of precipitate was proportionately greater.
- (c) The most sensitive test for ergosterol in these preparations appears to be the determination of the changes in specific absorption, when subjected to further radiation. Certain of our solutions, which gave no other evidence of the presence of ergosterol, showed an increase of several per cent. in absorption at wave-lengths 274 and 311 during the first few minutes of such radiation, and only after 5 minutes' radiation showed the fall in absorption directly proportional to time of exposure, such as would be expected if substance A were present alone. We suspect therefore that some of our preparations have been slightly contaminated with ergosterol. Such contamination would merely decrease slightly the absorption of the solution concerned.

Evidence indicating that Substance A is Vitamin D.

We believe that substance A is vitamin D for the following reasons, but must point out that no evidence of identity can be conclusive until either vitamin D or substance A has been isolated in an obviously pure state. The only characteristic by which as yet we can recognise vitamin D with certainty is its power of curing experimental rickets when given in very minute amounts. The attribution to this substance of any other characteristic, such as a definite physical or chemical property, can always be challenged by the supposition that the second property is due to another substance, which resembles the vitamin in its chemical properties sufficiently closely to resist separation or independent destruction in the processes used by the observer. Examples of this type are common among radioactive elements, and, pending final isolation of a substance, it is impossible to exclude such a possibility with certainty.

1. In a long series of quantitative comparisons of absorption spectra and antirachitic activity in solutions of substance A (from which ergosterol had been removed), we have found a roughly linear relation between the antirachitic activity and intensity of absorption at wave-lengths between 310 and 270, high activity being accompanied by high absorption, and decreased activity by proportionately lower absorption. On further radiation of such solutions both properties have decreased in the same proportion. Fig. 3 shows all the tests of this relation that we have made in which the rats used

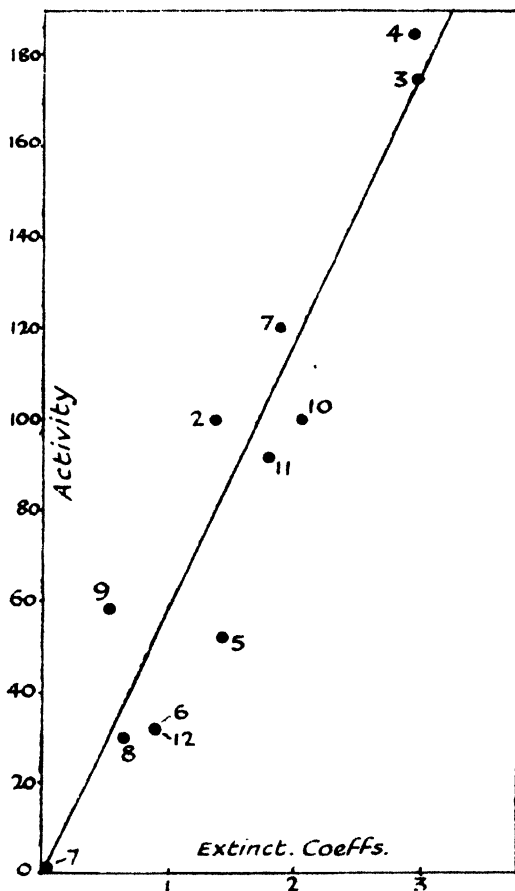


Fig. 3.—Relation between absorption of Substance A at wave-length 275 and antirachitic activity. Numbers refer to solutions in following list.

No.

Method of Preparation of Solutions Plotted in fig. 3.

1. Preparation of substance A from ergosterol radiated in alcohol for 1 hour.
2. Preparation of substance A from ergosterol radiated in alcohol for 10 minutes and later heated to 100° C. in oxygen for 10 minutes.
3. Preparation of substance A from ergosterol radiated for 30 minutes and later heated to 76° C. in nitrogen for 30 minutes.
4. Preparation of substance A from ergosterol radiated in hexane for 10 minutes.
5. Preparation No. 1 after a further radiation of $\frac{1}{2}$ hour through a cobalt chloride filter as described in text.
6. Preparation No. 1 after a further radiation of 2 hours through a cobalt chloride filter.
7. Preparation No. 1 after a further radiation of 18 hours through a cobalt chloride filter.
8. Preparation of substance A from ergosterol radiated in alcohol for 10 minutes. Re-radiated through filter.
9. Total products of radiation of ergosterol in alcohol for 5 minutes. Absorption corrected for unchanged ergosterol as described.
10. Total products of radiation of ergosterol in alcohol for 30 minutes. Absorption corrected for unchanged ergosterol.
11. Total products of radiation of ergosterol in alcohol for 1½ hours. Absorption corrected for unchanged ergosterol.
12. Total products of radiation of ergosterol in alcohol for 5 hours. Absorption corrected for unchanged ergosterol.

gave concordant results for any one solution,* and includes solutions treated by widely varying processes as described below the figure. It is obvious that the experimental errors are considerable. This is largely due to uncertainty in the biological estimation of antirachitic activity, which probably has an error exceeding ± 50 per cent., even when restricted to litters which have not shown obvious discrepancies. In view of this uncertainty a wide "scatter" must be expected, but it is evident from the general distribution that there is high correlation between the two properties, and that a direct linear relation is probable.

2. The most direct tests of this relation were conducted as follows: A solution of substance A prepared as described above was diluted with alcohol to 0.01 per cent., and then exposed in a silica cell with nitrogen stirring to radiation from which wave-lengths shorter than 265 were excluded by a "light filter" of alcoholic cobalt chloride. This caused the rapid destruction of substance A by conversion into substance B, which latter, having little absorption for wave-lengths longer than 250, was partly protected by the filter, and produced in considerable concentration. At intervals a sample was withdrawn for tests of absorption and antirachitic power. The absorption curves of a typical experiment of this type are given in fig. 4. The following table shows the estimated antirachitic activity and the relative heights of the absorption bands at 2747 in this series.

Solution.	Duration of radiation through filter.	Relative absorption at 2747.	Relative antirachitic activity.
F 17 <i>f</i>	0 hours	100	100
F 17 <i>l</i>	$\frac{1}{2}$ hour	67	54
F 17 <i>p</i>	2 hours	47	26
F 17 <i>r</i>	18 hours	1.9	0.05

While the relation between the two is only approximate, the extremely low activity of F 17 *r*, which was only detected by giving doses 200 times as great as those of solution F 17 *f*, is a direct proof that substance B is not vitamin D, and disproves the assumption made by earlier workers that the substance, produced during the radiation of ergosterol, and showing high absorption at 240, was vitamin D. We have repeated this experiment three times in all

* One preparation made by a modified process and showing abnormal absorption and a yellow colour has been omitted. The extinction "coefficients" in this diagram were inadvertently reduced one-tenth.

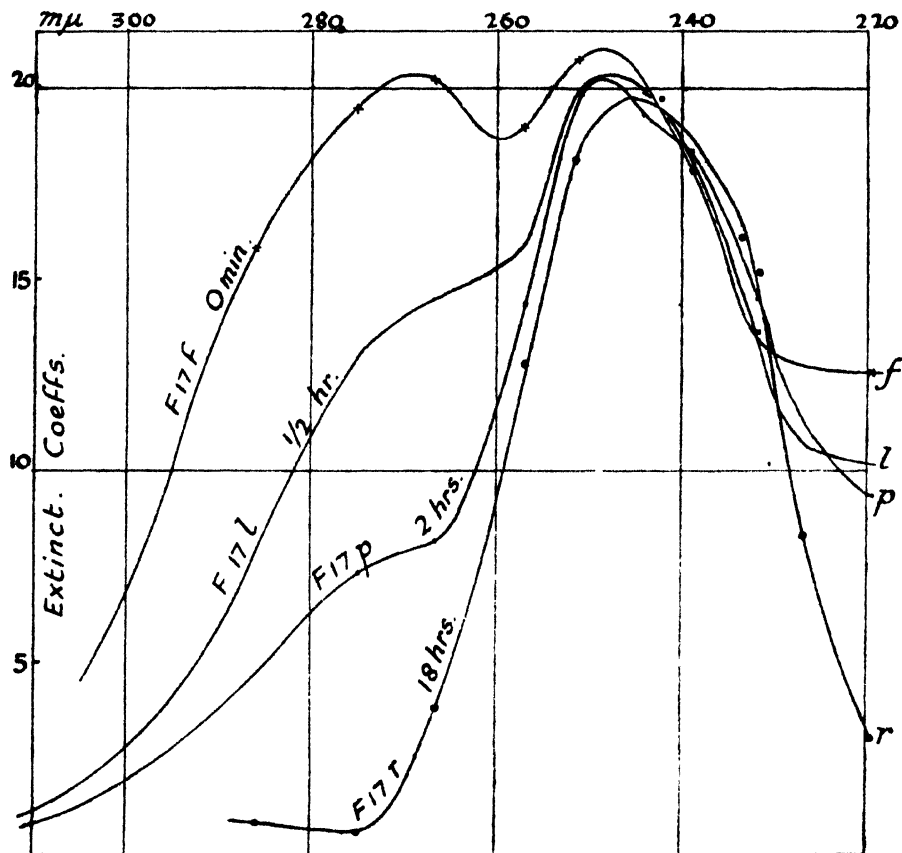


FIG. 4.—Effect of further radiation through cobalt filter, for times shown, on absorption of preparation of Substance A radiated for one hour before removal of ergosterol.

with similar results. In fig. 4 it will be noted that the original solution depicted, F 17 *f*, differs considerably in absorption from the substance A shown in fig. 2. This is because the original solution of ergosterol was radiated for 1 hour (before removal of unchanged ergosterol) in the preparation of F 17 *f*, and, therefore, considerable quantities of substance B were present before the start of the cobalt chloride radiation. The gradual transition of the curves from the type shown in fig. 2 to that of F 17 *f*, and thence to F 17 *r*, has been observed in other experiments, and suggests that under the influence of ultra-violet radiation substance A changes directly to substance B. The curve for F 17 *r* probably resembles closely the absorption curve of a solution of pure substance B.

3. Solutions of ergosterol have also been radiated for various times, and then divided into two portions. In one portion the absorption and antirachitic

activity have been measured without previous removal of ergosterol. In the other, the percentage of unchanged ergosterol has been estimated gravimetrically by precipitation with digitonin. Fig. 5 shows the percentage of

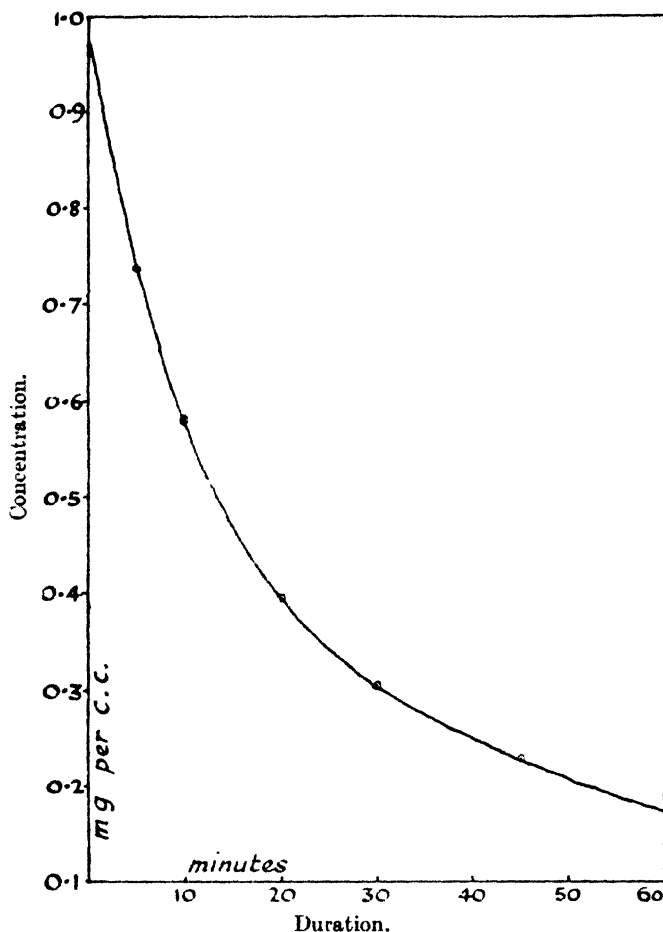


FIG. 5.—Concentration of unchanged ergosterol after radiation in alcohol.

unchanged ergosterol thus determined in the solutions whose absorption is shown in fig. 1. For each of the mixed solutions thus studied it is possible to calculate how much of the absorption at any wave-length was due to the unchanged ergosterol. By deducting this absorption from the value observed for the mixed solution we can calculate the absorption of the products of radiation. If the figures thus obtained for a solution which has only been radiated for a short period are plotted, the absorption curve thus produced is similar to the curve for substance A, shown in fig. 2, except for a proportionate

reduction in the height of the curve due to the reduced amount of substance A present. This similarity supplies further confirmation of the fact that the digitonin separation process does not introduce into the preparations of substance A impurities in quantity sufficient to show strong absorption, and also gives evidence that our preparations of substance A contain all the substances showing marked U.V. absorption that are formed in the early stages of the irradiation of ergosterol. Fig. 6 shows the absorption thus calculated for one

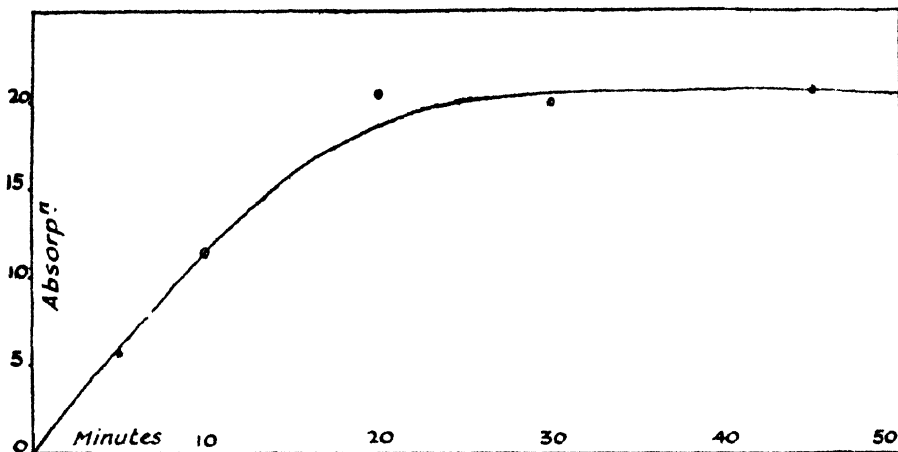


FIG. 6.—Absorption of radiation products of ergosterol at wave-length 275 due to Substance A only.

wave-length only of the products of radiation present in the series of solutions, for some of which the total absorption is shown in fig. 1. Since at these wave-lengths the absorption of substance B is negligible, this curve should give a measure of the concentration of substance A present in the mixtures. Tests of antirachitic activity done on these, and on other solutions radiated for longer periods, showed that the antirachitic activity was roughly proportional to the concentration of substance A thus determined, in that both showed a very rapid rise in the first 10 minutes, a slow rise after 20 minutes, a very flat maximum at about 40 minutes, and then a fall, much slower than the original rise, to about half the maximum in 5 hours, and negligible values in 60 hours. This evidence amounts to the statement that vitamin D and substance A are formed at a rapid and approximately equal rate, and are destroyed at a much slower, but also approximately equal rate under the conditions of our experiments. The rapid rise, flat maximum and slow fall fit well with the observations of Rosenheim and Webster (8), but are in contrast to the steep, narrow maximum found by Bills (7). The shape, however, of these curves showing

the change in absorption after different periods of radiation. depends so much on the exact conditions of radiation, that it is very difficult to get concordant results in different experiments, and there is no reason to expect agreement between the results of workers who radiate under different conditions.

4. Attempts have been made to destroy substance A by oxidation. and to observe whether the rates of disappearance of absorption at 280 and of antirachitic activity were equal. The effects produced by bubbling oxygen through alcoholic solutions of 0.2 per cent. strength at 76° C. for 1 hour (with subsequent addition of alcohol to restore the volume lost by evaporation) were too small to determine with certainty. Evaporation to dryness and exposure of the residue to gaseous oxygen at 100° C. produced a much more rapid effect, leaving a pale-brown solid residue. of much reduced solubility in alcohol. and with reduced antirachitic activity and reduced absorption at 280. The shape of the absorption curve of this residue is shown in fig. 7. This shows that both

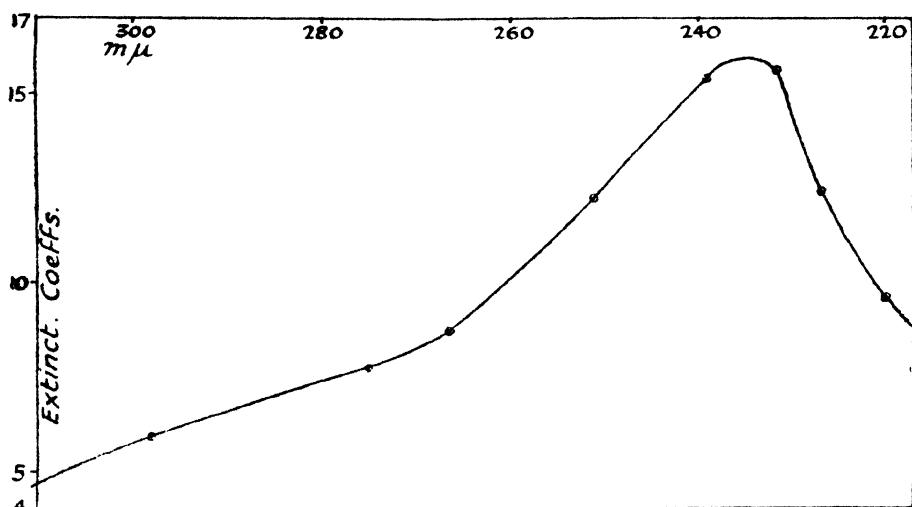


FIG. 7.—Absorption of mixture obtained by oxidation of Substance A.

substance A and vitamin D offer marked resistance in alcoholic solution to oxygen at 76° C., but that, when dried and subjected to oxygen at 100° C., both are oxidised with considerable speed.

5. We have never in our experiments obtained a solution showing strong absorption at 280 (in absence of ergosterol or pigment) that did not show strong antirachitic activity, or, conversely, a solution of high antirachitic power without absorption at 280. This evidence, like all that hitherto given as to the probable identity of vitamin D and substance A, in no way excludes the

possibility that vitamin D is a substance produced and destroyed simultaneously to substance A, but independently of it.

6. The following argument, though less direct as evidence, is not so open to this objection. In preparations of substance A the antirachitic activity is destroyed fairly rapidly by radiation of wave-lengths between 330 and 260, as follows from experiments in which we have found rapid destruction by radiation filtered through cobalt chloride, but only very slow destruction by similar radiation filtered through window-glass. Unless some indirect mechanism of destruction is assumed, this shows that vitamin D has an absorption band within these wave-lengths. Further, it is possible to make certain inferences about the structure of vitamin D from the apparent impossibility of forming it *in vitro* from any substances other than ergosterol or its esters. Attempts to produce it from numerous sterols allied to ergosterol have failed (8), and the addition of two hydrogen atoms to one of the three double bonds in ergosterol, forms a substance which cannot be activated. These and other facts obtained by the extensive researches of Windaus and his colleagues on the chemistry of vitamin D (9) make it very probable that vitamin D contains the three double bonds known to exist in ergosterol. But, if so, it is almost certain that it must show strong absorption of radiation at some wave-length between 200 and 800 $m\mu$.

Hence on the assumption that our preparations of substance A are a mixture of one absorbing substance with other non-absorbing products, it is probable that the absorbing substance (which we call A) is vitamin D. The most obvious alternative is the hypothesis that vitamin D is a substance of enormous biological activity, which is present only in a small percentage in our preparations of substance A, and hence, in spite of a high specific absorption, only contributes a small part of the total absorption. This seems improbable, since our best preparations of A are some two to four times as strong as a preparation of which the biological action can easily be detected in doses of 1/100000 of a milligramme per day, and consequently the activity of such a hypothetical substance would need to be very high indeed.

7. When ergosterol is radiated under the conditions we describe, the absorption increases regularly from the start, and in the earliest stages the increase is nearly proportional to the time of radiation. This shows a direct transition from ergosterol to substance A. The changes in absorption of substance A, when radiated in solutions free from ergosterol, provide a similar argument for the direct transition from A to B. This makes it improbable

that vitamin D is formed as an intermediate stage in the conversion of ergosterol to substances A, B and C, but does not exclude the possibility of its formation directly from ergosterol by an independent reaction.

Conclusions as to Relationship of Vitamin D and Substance A.

There is thus good reason for believing that vitamin D has an absorption spectrum very closely resembling that shown in fig. 2. It is, however, possible that substance A is merely a by-product formed at the same time as vitamin D, or that vitamin D and substance A are two closely similar substances related by some reversible process, such as condensation or polymerisation.

Estimate of Absolute Magnitude of Absorption Coefficients of Substance A.

Hitherto our preparations of substance A have been the colourless, glassy masses described previously (2), and we have failed to crystallise or otherwise purify it. It was therefore uncertain whether our preparations contained only a small quantity of a substance of very high specific absorption, or a relatively large quantity of a substance of lower absorption. The question is of some interest, since estimates of the absolute activity of vitamin D have hitherto depended on assumptions as to quantum efficiency of doubtful validity (10).

The method described below is based on the following assumptions:—

1. That ergosterol when radiated changes directly into substance A, and does so without the loss of any large part of its molecule, so that no great change of weight is involved. This is very probable for the reasons given above.
2. That substance A is vitamin D. If it is not, the argument holds for substance A, but not for vitamin D.
3. A further assumption is made which is of doubtful validity, but which only affects a correction factor amounting to 20 or 30 per cent. of the magnitude concerned. Namely, that in the destruction by radiation of two solutions, of which one contains substance A only, and the other a mixture of A and ergosterol, the rate of destruction of A in the second solution would be reduced by a fraction equal to the ratio of the absorption of the ergosterol present to the absorption of the mixed substances. This assumption could only be exactly true if the ratio of absorption of photochemically active radiation by ergosterol and substance A were constant for all wave-lengths. This is approximately the case, as can be seen by the close similarity of the curves in fig. 2.

4. That in alcoholic solution no reaction occurs between ergosterol and substance A.

The method depends on equating the rate of destruction of ergosterol (determined gravimetrically) by radiation, with the rate of production of "absorption due to substance A." Consider the solutions of which some properties are shown in figs. 1, 5 and 6. After 20 minutes' radiation the tangent to the curve in fig. 5 shows that ergosterol is being destroyed at the rate of 0.012 mg. per cubic centimetre per minute. On the assumption that ergosterol is being converted directly into substance A without material loss of weight, this shows that substance A is being formed at the same rate of 0.012 mg. per cubic centimetre per minute. Now the rate of net increase in "absorption due to substance A" after 20 minutes' radiation is given by the tangent to the curve in fig. 6 as 0.35 units per minute.* This rate of net increase is equal to the difference between the rates of destruction and of production of absorption, *i.e.*, the rate of production is equal to the sum of the rates of net increase and of destruction. The latter rate was determined as follows: A quite independent experiment was done in which a preparation of substance A of 0.1 per cent. concentration was radiated under identical conditions of rotation of cell and illumination. Photographs taken from time to time showed a mean rate of destruction of "absorption due to substance A" of 0.48 unit per minute. We can now calculate the rate of destruction of "absorption due to substance A" (*i.e.*, the rate of total reduction of absorption which would occur if no further substance A was being produced) in a mixed solution of ergosterol after 20 minutes' radiation, by making a correction as follows for the active radiation absorbed by the ergosterol. The percentage of ergosterol left unchanged at this point was 38; the absorption due to this at wave-length 2747 would be 7.6 units; the total absorption at 2747 was 28 units. Therefore, of the incident radiation of wave-length 2747, a fraction $= 7.6/28 = 27.1$ per cent., was absorbed by ergosterol and therefore rendered unavailable for absorption by substance A. Since the fraction of other wave-lengths absorbed by ergosterol is closely similar to that of 2747 we can assume that substance A was only attacked by 72.9 per cent. of the radiation used, and was

* The unit of absorption employed in this paper is that which reduces the incident light to 1/10 of its intensity after passing through 1 cm. thickness of solution of concentration 1 gm. per litre. The rates of change therefore represent the changes in the extinction coefficient E , where $E_c = \log_{10} \frac{I_0}{I_1}$ for a layer 1 cm. thick, and I_0 and I_1 are the incident and transmitted lights, and c is the concentration in gms. per litre.

being destroyed at 72.9 per cent. of the rate observed in the test with a pure preparation of A. This equals $0.48 \times 72.9/100 = 0.35$ unit per minute. On adding this rate of destruction to the rate of net increase (0.35) found above we get a total rate of production of 0.7 unit per minute. Therefore the addition of 0.012 mg. per cubic centimetre of "pure" substance A causes an increase in extinction coefficient of 0.7 unit. Hence the extinction coefficient of a solution containing 1 gm. per litre of pure substance A would be $0.7/0.012 = 58.3$.

A similar calculation applied after radiation for 10 minutes gives a coefficient of 58.0. A similar calculation applied after 30 minutes' radiation gives a value of 60.2. This close agreement is probably fortuitous, since the experimental errors involved in the determinations concerned were considerable. It is possible to apply the calculation to any point on the curve shown in fig. 6, but as the initial end is approached the correction for absorption due to ergosterol becomes unduly large, and, since this correction is only approximate, the error increases. If points are selected after much more than 1 hour's radiation, the rate of destruction of ergosterol becomes unduly small, and the error of its gravimetric determination increases. Hence points between 10 and 60 minutes are the most reliable. The extinction coefficient thus found suggests that in our purest preparations of substance A the concentration is about 55 per cent. As already mentioned the smallest daily dose of our purest preparations which we should expect to detect by radiographic measurements of calcification would be about $1/400000$ mg., i.e., a total in 14 days of $14 \times 2.5 \times 10^{-9}$ gm. = 3.5×10^{-8} gm. If we multiply this by the above figure of 55 per cent. we get 1.9×10^{-8} gm. as the smallest dose of pure vitamin that we should detect. This is in almost ludicrously close agreement with the value (2×10^{-8} gm.) found by Fosbinder, Daniells and Steenbock (10) and by Coward (11), but the "minimum dose of vitamin that can be detected" is so greatly dependent on the exact condition of the rats employed, that calculations of this sort are of little significance.

Technique of Measurement.

1. *Spectrographic.*—The absorption measurements were conducted as follows: Light from a spark produced by a 10,000-volt 50-cycle current between one cadmium electrode and one of tungsten alloy, radiated directly on to the slit of a Hilger quartz-spectrograph. The upper half of this slit was covered by a cell of fused silica with optically polished walls 1 cm. apart. Owing to the presence of imperfections in the cell it was found necessary to move it to and

fro across the spectroscope slit throughout each exposure. The exposures were uniformly of 20 seconds and were controlled by a slowly rotating sector. Thus each exposure gave two spectra, one through air, and one through the cell containing the solution under investigation. In order to correct for possible variations in " γ ," *i.e.*, in the slope of the intensity-density curve, each plate was calibrated by taking a photograph of the spark through a "neutral tint" quartz and gelatine wedge, placed vertically directly in front of the slit. A print from a typical negative is reproduced in Plate 36. The wedge was calibrated separately for each wave-length measured by two separate methods as follows: On one plate a photograph was taken through the wedge as usual, and other photographs were taken in each of which two sectors of different aperture were rotated about 100 times directly in front of the slit. Thus each of these photographs gave two spectra, of which one was due to 100 exposures of say $1/5$ second, and the other due to an equal number of exposures of, say, $1/20$ th second. By using a series of sector settings the times of exposure were varied over a range of 1 to 18. The different sector openings gave concordant results for the wedge gradient, thus showing that this was very nearly uniform for any given wave-length. The above method of calibration is closely similar to that used in the rotating sector photometers with which the majority of ultra-violet absorption work has been carried out. Most of these rotating sector methods make the assumption that Schwartzschild's constant p , in the equation $D = \log I t^p = i$, where D = density of plate, I intensity of light, t time of exposure, and i the "inertia" of the plate, is equal to unity. This assumption is of doubtful validity, and we therefore calibrated the wedge by another method in which the intensity of the incident light was reduced by perforated metal screens placed sufficiently close to a lens to throw a diffuse, nearly uniform image on the slit. This method gave rather variable results, owing to difficulty in getting a steady light source, but gave mean values for the wedge constant closely agreeing with the sector method for all wave-lengths between 240 and 290.

The densities of all negatives were measured on an accurate photoelectric microphotometer, designed after a modification of Dobson's pattern (12) and constructed for us by Dr. E. Schuster, to whom we wish to express our very great indebtedness. The error of measurement of density with this instrument was under 0.5 per cent. The errors due to uneven illumination of the slit and development of the plate probably amounted to less than 2 per cent., since the density of spectral lines measured at a series of points along their length varied by less than this. All plates were developed and fixed in a

nickel tank, with rapidly moving plunger as described by Dobson, Griffith and Harrison (12), for a uniform time at a temperature adjusted as close to 18° C. as practicable.

For wave-lengths longer than 250 the above method of calibration was satisfactory, and allowed of the direct comparison on each plate of points of approximately equal density on the wedge image and the other images. In practice the plates were found to be so uniform that a standard set of wedge-curves could be used for most plates after checking agreement on one wave-length only. This much reduced the labour of calculation. For wave-lengths shorter than 240, the method is unsatisfactory, owing to the rapid increase in wedge gradient due to excessive absorption by the gelatine. By 235 the gradient had risen to 6.4 per centimetre, and was so steep as to cause small errors in observation to produce large errors in the results. Hence, for wave-lengths shorter than 245 the plates were not calibrated directly, but were assumed to have the same values of " γ " as the plates which had been calibrated by the rotating sector. The reliability of this assumption depends on the uniformity of the emulsions and conditions of development. It was easily shown to be approximately correct for wave-lengths between 240 and 270, by plotting absorption curves from the same photographs by the two methods. It is, however, impossible to exclude errors due to variations in γ occurring in the short wave-lengths, but not in the longer ones, and for this and other reasons the values given for these short wave-lengths must be regarded as less reliable than the others. All absorption data given represent the absorption due to solute only, and have been corrected by deducting the absorption due to cell and solvent as measured in separate photographs. This correction is of serious magnitude (1) in the very low values recorded for wave-lengths 311, (2) in the values for wave-lengths below 230 where the absorption of solvent becomes serious. In these regions our curves for ergosterol continue to fall, solely owing to the application of this correction. If it is not applied our curves would rise from 225 to 215, as observed by some previous workers. The correctness of this solvent correction is shown even at 215 (where it is very large) by close concordance between values of absorption calculated from solutions of widely different concentration. This concordance provides a delicate and efficient safeguard against many of the errors which are likely to occur in photographic work, and in order to utilise it most of our solutions have been photographed in several dilutions.

2. *Biological.*—The biological tests were carried out as follows: Albino rats of age about 1 month, and weight 50–80 g., were placed on the diet 2965 recom-

mended by Steenbock (13) for from 10 to 13 days, and kept thenceforth each in a separate box. They were then anaesthetised by ethyl chloride, while an X-ray photograph was taken of the left knee-joint. The Steenbock diet was then supplemented by a daily dose of the substance under examination dissolved in olive oil. This dose, which was usually 20 c.mm. of solution, was given directly into the mouth by a capillary pipette. In most litters some of the rats were given doses of 1/5000, 1/10000, and 1/20000 mg. of a standard preparation of radiated ergosterol, which was kept in a concentrated solution in olive oil at -6°C ., and from which the dilutions actually employed were made fresh every 3 weeks. The remaining animals of each litter were given graded doses of the solution under test. In cases where exact comparison between two fluids was of especial importance, additional litters were used, in which the standard solution was replaced by one of the liquids under test.

Further X-ray photographs of the knee-joint were taken on the 14th day after the first dose of vitamin, and again on the twenty-first day, when the animals were killed. The diet used produced a fairly uniform degree of severe rickets, in which the length of wholly uncalcified space between the upper epiphysis and diaphysis of the tibia was about 1.3 mm. before the vitamin test began. In a few cases rats were used for test, with this space as low as 0.9 mm., but this was exceptional and noted in assessing the results.

The degree of healing was estimated as follows: A standard scale of photographs was prepared, comprising 14 degrees of healing, from 0 which showed no calcification, to +13 which showed almost perfect healing. All these photographs were obtained from rats, photographed on the fourteenth day after first dosing with vitamin, under the conditions described above. Each photograph of a rat under test was compared with the standard scale of photographs, and allotted the number which most closely corresponded to the degree of healing shown. When the same photographs were assessed by different observers the concordance was almost always within ± 1 . Thus the standard scale can be said to give a reliable division into six different degrees of healing and a seventh degree of no healing.

The series of rats by which any one fluid was tested was then arranged in a table, such as the following example, which represents the result of the biological test for the fluid F 26 b.

Daily dose of substance.	Degree of healing with standard preparation.	Degree of healing with preparation F 26 b.
mg.		
1/5000	11	11
1/10000	8	8
1/20000	6	7

Conclusion.—F 26 b has approximately equal activity to the standard solution.

The final estimate of activity was then made by assessing the various pairs of rats concerned. To aid in this a mean value was determined for the number of units on our scale, corresponding to a change of 100 per cent. in the dose of standard solution used. It was found, on taking the average of a large number of litters, that a dose of 1/20000 mg. gave figures approximately 2 units lower than a dose of 1/10000 mg., and this dose gave figures about 2 units lower than the dose of 1/5000 mg.

In assessing preparations of strength different from the standard solution, large or small amounts of the test substance were given according as high or low activity was expected. As a rule, however, the mean results for a test solution were from 1 to 3 units higher or lower than those for the standard solution, and their difference was assessed by using the figure of 2 units per 100 per cent. change found above.

In a large percentage of our litters the results obtained were somewhat discordant, owing apparently to differences in the sensitivity of the individual rats rather than to errors in the technique of dosing or estimation of healing. The results obtained from litters that showed obvious discordance between members of the same litter have been excluded from fig. 3. We have, however, plotted a scatter diagram showing the results for all the fluids of which both activity and absorption were measured. This diagram appeared closely similar to fig. 3 except for a wider scatter.

Preparation and Radiation of Solutions.—The ergosterol used for most experiments was kept in small sealed evacuated tubes opened shortly before use. It showed m.p. 163° and an optical rotation in 1 per cent. solution in chloroform $\alpha_{5461} = 152^\circ$.* Another especially purified sample used for some experiments was dried *in vacuo* over phosphorus pentoxide and then gave $\alpha_{5461} = \text{approximately } 135^\circ$, and gave closely similar results on radiation. Solutions were made up as far as possible immediately before use in solvent

* The molecular extinction at 282 $m\mu$ was 10,800.

which had been freshly boiled in order to expel dissolved oxygen. While air was not entirely excluded from solutions, nitrogen was used when practicable.

All radiation was carried out in silica cells of internal thickness 1 cm. at about 6 inches from the K.B.B. mercury arc, as described early in this paper, or at 10 inches from a vacuum mercury arc run at 3 amp. and 130 volts. Under these conditions the two light sources gave approximately equal currents in a photoelectric cell with sodium kathode and quartz window. During radiation the space above the solution concerned was always filled with nitrogen.

Summary.

The action of ultra-violet radiation on ergosterol has been studied by comparing the absorption spectra and antirachitic activity of the products formed. It is concluded that three substances (or groups of substances) are produced in succession, of which the first shows an absorption band roughly similar to that of ergosterol (maximum 280 $m\mu$), but more than twice as intense. This absorption is accompanied by great antirachitic activity, and evidence is given showing that the substance concerned is probably vitamin D.

The second product, which is formed by further radiation of the first, shows a strong absorption band with maximum at 240 $m\mu$, and has no antirachitic activity. The third substance is formed by further radiation of the second, and shows neither antirachitic activity nor marked absorption.

A method of calculation is described by which the actual percentage of vitamin D present in the purest preparations studied is estimated as over 50. Absorption curves are shown which are believed to be of closely similar shape to those which would be shown by pure preparations of vitamin D and of the second product of radiation.

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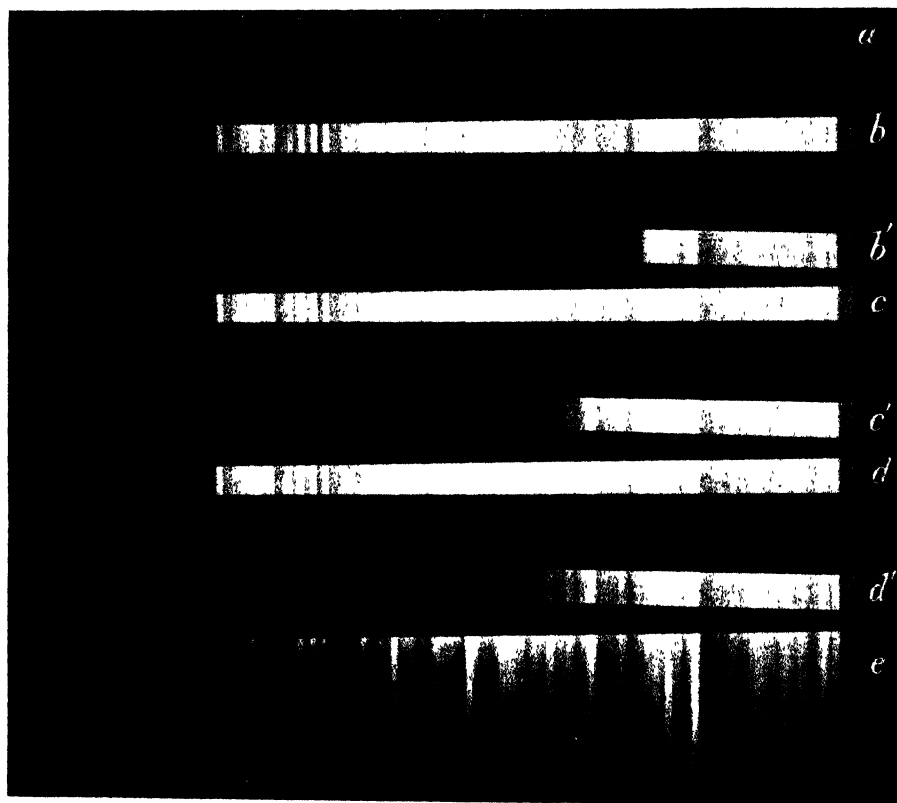


FIG. 8. —Print from negative showing absorption of preparation of substance A.

a—Approximate wave-length scale—for illustration only

b, air
b', solution 0·1 per cent. } First photograph.

c, air
c', solution 0·01 per cent. } Second photograph.

d, air
d', solution 0·005 per cent. } Third photograph.

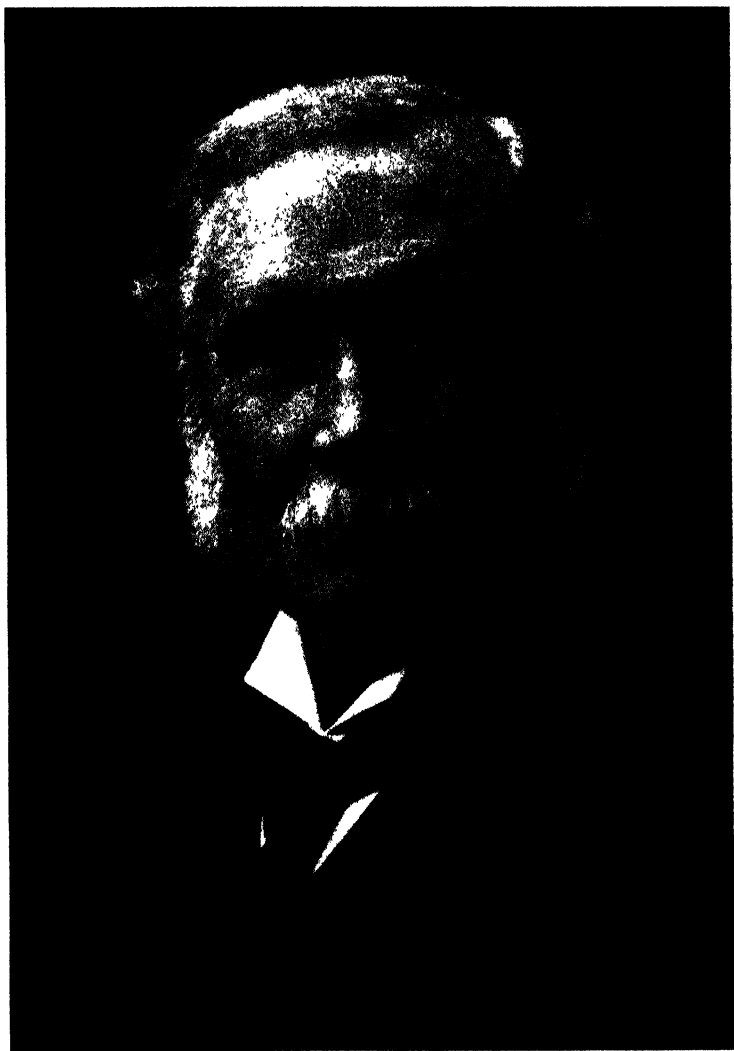
e, image of wedge used for calibration.

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OBITUARY NOTICES
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John Horne

JOHN HORNE—1848–1928.

WITH the death of Dr. JOHN HORNE on May 30, 1928, one of the most distinguished and certainly the most beloved of Scottish geologists of the last generation, has passed to his rest. Horne had reached the ripe age of 80 years, having been born in the Stirlingshire village of Campsie on the first day of January, 1848. He was educated at the High School of Glasgow and subsequently attended classes at the University. He did not graduate, and it was not infrequently the case that students at that time did not go up for a degree. Both Archibald Geikie and James Geikie studied at Edinburgh University but neither of them sat for degree examinations. Probably the reason was partly that boys of strong individual bent took a variety of classes that were outside the ordinary curriculum. Already at the age of 19 Horne had developed an interest in geological work and was accepted as a recruit for the Geological Survey of Scotland in 1867. Appointment in those days was largely a matter of recommendation and personal selection, and the appointment in this case was amply justified.

The Scottish Survey staff at that time included in its members, Archibald Geikie, James Geikie, and Benjamin Peach, and they were at work mostly in Fifeshire, Ayrshire and Dumfries-shire. Horne spent a considerable time on the Silurian rocks of Dumfries and Kirkcudbright, a series which was then little understood, but which he was to revise at a subsequent date in a piece of work that was one of the greatest achievements of his scientific career.

Horne rapidly showed himself a geologist with a good eye for country. He was also an exceptionally neat mapper, as his field copies show very clearly. But the work he was doing, though good training, offered very little scope for original research. The time was not ripe for an explanation of the sequence and tectonics till Lapworth revealed the meaning of the graptolite zones. The record of Horne's work at this period consists consequently of the official maps of the ground he surveyed and the brief explanations that were published to accompany them.

His natural inclination rapidly developed in the direction of tectonics and stratigraphy; and he was especially attracted by glacial problems, a subject that was then very keenly debated and which was making extraordinary progress. As official publications in those days were few and unsatisfactory we find that he and his colleagues spent much of their holiday time in investigations that could be published extra-officially. His first appearance in this rôle was in a paper on the Isle of Man printed by the Edinburgh Geological Society in 1874. Horne was a most companionable man, and the band of workers among whom he was placed included Sir Andrew Ramsay, Archibald Geikie, James Geikie,

Benjamin Peach and James Croll, all of them intensely interested in glacial problems and all working very actively at that subject. His friendship with Peach, which was to develop into a life-long partnership, one of the most celebrated and most productive in the annals of British science, was started when he joined the service, as Peach had been his first companion in the field. Peach knew well that many interesting problems awaited solution in the north of Scotland as he had lived for some time in Wick, and, joined by Horne, he made tours in Shetland, Orkney and Caithness in 1876, 1880 and 1881 that resulted in a series of very valuable papers that are still the foundation of our accurate knowledge of the geology of these districts. As became geologists of wide experience they neglected no part of the field. Sedimentary rocks, volcanic rocks, tectonics and glaciation were very fully investigated and described. The most important result was the clear proof that this part of north-east Scotland was glaciated by ice that came out of the basin of the North Sea and passed in a westward and north-westward direction across Shetland, the Orkneys and Caithness. They also detected evidence of a local glaciation in the higher valleys. These results confirmed the earlier observations of Peach's father, and Robert Dick, and the speculations of James Croll. They also corrected the inferences of Jamieson who had mistaken the direction of ice movement and regarded the boulder clay as a marine deposit. Peach and Horne made also some useful discoveries regarding the geological structure of the Orkney archipelago, and in Shetland their researches revealed for the first time the rich variety and extraordinary interest of the Old Red Sandstone volcanic and intrusive rocks that make some of the most striking coastal scenery in Great Britain.

In the meantime the Geological Survey of Scotland had realised the necessity of tackling a problem of great difficulty that was being ardently discussed by many distinguished geologists. Sir Roderick Murchison and Sir Archibald Geikie had formulated an explanation of the structure of the North-West Highlands that had aroused much criticism. The alternative views of Nicol had many supporters, and the new work that was being done on Alpine tectonics had awakened intense interest in the action and effects of mountain-building forces. Horne, having completed his work in Dumfries-shire, had been transferred to Nairn and Inverness in 1877, and by 1883 had mapped a considerable area of metamorphic rocks and Old Red Sandstone in the basin of the Moray Firth. When it was decided to open the survey of the North-West Highlands he was taken from that region and sent to Durness with Peach. Lapworth had been already at work on that ground, but was suffering from ill-health. Peach and Horne worked independently of Lapworth but after a few months it was clear that they had arrived at the same conclusions. Sir Archibald Geikie was promptly informed and went north to inspect the sections. He found that the evidence was good enough to carry conviction. He frankly

recanted his previous conclusions and gave a free hand to the field geologists both in the surveying and in the publication of the results. The first announcement of this change of front was made in a letter to 'Nature' of November 29, 1884, in which it was clearly stated, that the "Eastern Schists," now known as the Moine Schists and Gneisses, did not conformably overlies the Cambrian Series, and that the present relations of these rocks had been determined by a powerful series of dislocations or thrusts by which the crystalline gneisses had been forced westwards and piled upon the fossiliferous Palæozoic sediments. The controversy which had lasted for many years was now at an end, and it was possible to attain a clear conception of the geological history and structure of the North-West Highlands that would be generally accepted.

The staff that worked with Peach and Horne in this country were Gunn, Clough, Cadell, Greenly and Hinxman. The district proved to be one of exceeding complication, but fortunately the component rock systems were well defined by their individual characters and were easily separable. Attention was confined to the narrow belt of thrusting and the Lewisian rocks that lie to the west of it. The Eastern Schists were left to be worked out at a later time. No serious differences of opinion arose between the surveyors and the mapping proceeded steadily. The Annual Reports of the Director showed the progress of the work, and in five years it was apparent that a large amount of new information had already been gleaned. A condensed statement of the principal results and conclusions with an outline of the evidence was read to the Geological Society of London in 1888. It was drafted by Horne and has long been recognised as a masterly statement of a very intricate subject, which left no doubt as to the strength of the evidence or the validity of the conclusions arrived at.

Among this band of workers Horne was definitely the organiser. His long experience and sound judgment together with his genial personality, diligence and methodical habits eminently qualified him for the task. His enthusiasm was unbounded and till his last day he referred with pride to the Moine Thrust-plane as the most wonderful geological structure of his native country, so powerful, so clear and so convincing that one glance was sufficient to dispel the doubts of the most sceptical. From the first he worked in very close association with Peach, in fact some of the most intricate ground was done by them jointly. This partnership was in every way ideal, for each had certain strongly marked characteristics and capacities, which in the other were less highly developed. The brilliant imagination and profound speculations of Peach were balanced by the sound judgment and clear logical deductions of Horne. Peach was a rapid worker, of far-sighted vision, who arrived at conclusions almost before he had finished collecting the evidence. Horne was a man of very judicial mind who could bring into one focus converging rays of evidence from a wide field, and who arrived at no conclusions till all the facts

had been given their proper weight. Peach's knowledge of palæontology was of the highest value. Horne specialised rather in tectonics and the preparation of irreproachable maps. Both were men of splendid physique and of inexhaustible energy. The result was a series of maps that have never been excelled for clearness, detail and completeness. These were glorious hours which both looked back upon with fond recollection. The remarkable structures which they deciphered were in those days full of novelty and interest. In this particular department the science of geology has made great progress since that time, but though those districts have been visited by many distinguished geologists from all parts of the world no flaw has been detected in the field work done by Peach and Horne, and criticism has been confined to minor details of interpretation and correlation.

By 1897 the whole of the Lewisian Gneiss of the North-West had been mapped, extending nearly 100 miles from Cape Wrath to the Kyle of Lochalsh, and the Moine Thrust-plane had been traced through its whole extent on the Scottish mainland. Many of the maps had been published and the time had arrived to put forth a statement of the scientific results that had been achieved. This was a heavy task, for though each geologist undertook the description of his own district, the condensation and editing of the report involved many months of labour. The onerous duty fell upon Horne, who fortunately had all the necessary qualifications. He knew more about the whole area than any other geologist, and he had a crisp literary style that enabled him to present the subject with perfect clearness within reasonable limits of space. He was splendidly supported by Peach, whose artistic faculties were of a high order. In addition to contributing the palæontology Peach drew a beautiful set of sections across the country that make the structures as clear as in a model of transparent glass. Fortunately also Teall was at hand to do the petrology, and the final touches were given to the memoir by the skilled editorial hands of Sir Archibald Geikie. The result was the well-known volume on "The Geological Structure of the North-West Highlands of Scotland" which is recognised as a classic of British geology.

Meanwhile the work of Lapworth had called attention to the necessity of a revision of the mapping of the Silurian rocks of the South of Scotland. By means of the graptolite zones it was now possible to identify numerous horizons in that great series of rocks and to unravel the complicated structure. Lapworth had studied and mapped certain areas with inimitable thoroughness and detail. A general revision of the whole area, however, was urgently needed. This was undertaken by Peach and Horne, who, using the 6-inch geological maps, visited every place where graptolitic shales were known to occur and made very elaborate new surveys. The manner in which the work was done strikingly illustrates their energy and capacity. Engaged in Highland mapping during most of the summer, they returned to Edinburgh in the

autumn and paid short rapid visits to all the known exposures of fossiliferous Silurian rocks in such time as could be spared from other activities. In this case also Peach prepared the diagrams and sections and determined most of the fossils. To Horne was entrusted the completion of the maps and the descriptive accounts that appeared in the memoir. Six years or more were required for the work, which was finally published in 1899 under the title "*The Silurian Rocks of Britain, Vol. 1, Scotland.*" The fullest acknowledgment was made of the pioneer work of Lapworth, whose researches in this district are fundamental. One of the principal results of Peach and Horne's revision of these maps was the discovery of the widespread occurrence of a horizon of Arenig pillow-lavas accompanied by beds of radiolarian chert.

In those periods of the year when work in the North-West Highlands was impossible owing to sporting rights or to the inclemency of the weather, Horne continued his surveying in Nairnshire and Inverness-shire, and also, along with Peach, mapped an extensive area along the north coast of Sutherlandshire. In the latter district he was accompanied by Greenly, and in 1896 they published an important paper on Foliated Granites in East Sutherland which showed that they were fully alive to the strange problems presented by the metamorphism of the Eastern Gneisses and their permeation by granitic intrusions. Although several of the maps of these districts were published the memoirs descriptive of the field work were left to a later time. During these years Horne must have been intensively active as every season he was at work in the North-West and the North-East Highlands, and in the Silurian rocks of the South of Scotland. The winter was mostly spent in Edinburgh where the preparation of his more important contributions to the literature of Scottish geology had the first claim on his energies.

In 1901, on the retirement of Sir Archibald Geikie, there was a considerable reorganisation of the Geological Survey and Horne was appointed Assistant Director in Edinburgh with charge of the work in Scotland. It is characteristic of his unselfish nature that he did all he could to persuade Peach, who was his senior in service, to accept the post, promising him his loyal support, but Peach knew that the duties would be distasteful to him and he declined. There is no doubt that a better choice than Horne could not have been made. For experience, sympathy and administrative ability he had no equal, and moreover he thoroughly enjoyed the task. Much remained to be completed and published of the work done in former years, and new responsibilities were forced upon him as a revision of the coalfield maps was decided upon and a staff had to be trained in these exacting duties. Horne had never had much experience of coalfield mapping, but to a man of his capacity this presented no great difficulty, and in a short time he had initiated the new series of coalfield publications which are models of their kind and have been received with universal approbation. Absence in the field for prolonged periods of surveying was now

no longer possible, but he made frequent visits of inspection and kept closely in touch with all new discoveries. His pre-eminent qualities as a judge of field evidence now became more valuable than ever and many difficult problems were submitted for his decision. He had a very cultivated mind and had read widely in the geological literature of foreign countries; consequently his verdicts were usually final. But native caution was strong in him and his overflowing sympathies made it impossible for him to speak a hard word or issue a harsh verdict. Moreover he had seen so many established conclusions overturned and discarded that his mind was open to all suggestions. These field visits often took the form of open discussions, and if Peach were present, with his ingenious hypotheses, and some other experienced field men like Clough and Hinxman, with a wide knowledge of Scottish geology, the experience was sure to be a delightful one for young geologists fresh from University studies and comparatively untrained in the perplexing interpretation of field evidence.

Much of his time was occupied in the editing of maps and memoirs, and with Peach he contributed an excellent sketch of the geology of Scottish lakes to Murray and Pullar's "Bathymetrical Survey of the Scottish Fresh-Water Lochs." These two also wrote a valuable paper on the Canonbie Coalfield. After the publication of the North-West Highlands memoir in 1907, however, his functions became more definitely editorial and administrative. There was enough of this work to occupy all his time. His services also were constantly in request by scientific societies, and the number of committees which he attended was extraordinary. No scientific movement which was allied to geology or geography failed to secure his sympathy and support, and as he was known and admired by a vast body of scientific men the calls upon his time and services were very numerous. The societies in which he took most interest were the Royal Physical Society of Edinburgh, the Geological Societies of Glasgow and Edinburgh, the Royal Scottish Geographical Society and the Royal Society of Edinburgh. He served on the Councils of all these Societies and in due course occupied the presidential chair. Of the Royal Society of Edinburgh he was always a warm supporter, and he made a very charming and distinguished President during the years 1915 to 1919. The British Association's visits to Scotland also found in him a most efficient organiser and contributor. He served as President of Section C at the Glasgow meeting in 1901, and at the Dundee meeting in 1912 when Peach was in the chair he was the centre of all the activities both in the field and in the meeting room. After that meeting he and Peach led a 10 days' excursion to the North-West Highlands. He was editor of the 'Scottish Geographical Magazine' from 1915 to 1920. This kind of work he thoroughly enjoyed, as he was a ready writer and fluent public speaker. With a clear strong voice he could address a large audience and hold their attention closely, even on intricate scientific subjects. As a chairman of committees he was unexcelled, for his extraordinary tact

was combined with a shrewd knowledge of men and an excellent judgment of practical affairs. Consequently any proposal that he backed was assured of success.

These personal qualities more than his official position made him recognised as the leader of the Scottish geological world, and on the death of James Geikie and John Murray he was looked to as the chief promoter of all schemes of scientific character in geology and geography. He was also at this period frequently an examiner in geology in the Scottish and some of the English Universities. This gave him an opportunity of keeping in touch with many of the younger men who were doing research and looking forward to a scientific career, and his warm sympathies and wide knowledge were the best possible qualifications for work of that kind. No doubt his many activities cut largely into the time at his disposal, and comparatively little was published of his own investigations during this period. He was editor, however, of the 'Survey Memoirs' on the Edinburgh and Glasgow districts, and put a lot of work into these volumes. Many other memoirs passed through his editorial hands and he was always discriminative though seldom severe.

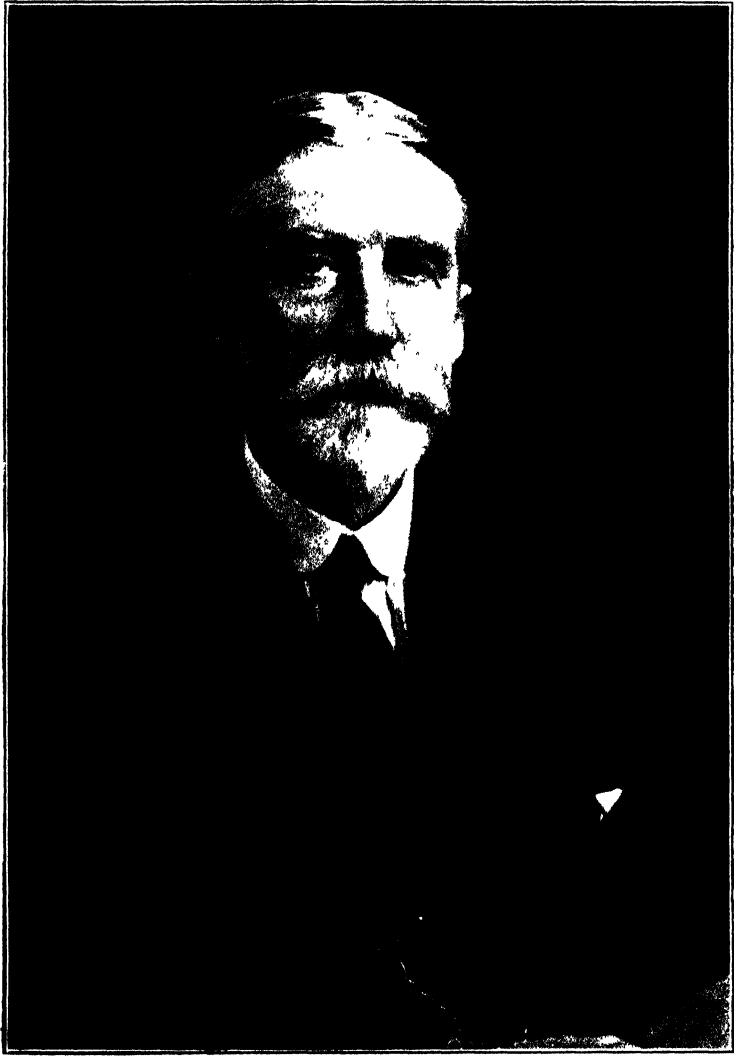
In 1911 he retired from the Geological Survey, but as he continued to reside in Edinburgh there was practically no interruption of his scientific work. He continued to collaborate with his former colleagues, and now found time to complete the descriptions of areas that he had surveyed many years before. A group of Highland memoirs received contributions from his pen: Fannich Mountains (1913), Lower Fimhorn and Lower Strathmairn (1923), Beaully and Inverness (1914), Mid-Strathspey and Strathdearn (1915), and he wrote, along with Peach, a brief handbook to the Geological Model of the Assynt Mountains. The maps of the South of Scotland and of the North-West Highlands were corrected and reduced to final form during this period. But the chief employment of his leisure was to be the preparation of a description of the Geology of Scotland which was to contain the ripened fruit of the studies and researches of a lifetime. No one who has read his address to the British Association in 1901 can fail to see his eminent qualifications for such a task. In this work Peach and he were to give the world their considered judgment on all the problems of Scottish geology along with the observations they had themselves made during a long career. There was hardly a parish in Scotland which they had not visited. For 50 years no advance in Scottish geology had been made of which they had not an intimate personal knowledge. The volume would also have contained much information that they had gleaned from the whole field but had never published and which no one else could possibly supply. This projected work was not completed at the time of his death; it is expected, however, that the chapters dealing with the Highlands will soon be published.

The ideal partnership between Peach and Horne, which lasted for 60 years

and marks an epoch in the history of Scottish geology, has practically no counterpart in the history of British science. Their work became so perfectly blended that no one could tell the part contributed by each. Each of them understood the other's mind so thoroughly that he knew what he would say before it was said. In the field with Peach it was amusing to find him building up some glittering speculation, and when he was finished he would remark "And now Horne would say, so and so." If in matters of theory Peach always went in front, it was Horne who followed and gathered up the results. Without Peach, Horne would have had a great scientific career. His industry and thoroughness, his fine discrimination, and the philosophic character of his mind, combined with his intense enthusiasm for the advance of knowledge would have brought him to the front in any subject to which he gave himself. Without Peach, however, something would have been lacking, for no one enjoyed more than Horne the brilliant flashes of intuition and imagination which Peach scattered around him. Peach was a constant stimulus to anyone who worked with him. Without Horne there is no doubt that Peach would have accomplished many brilliant feats. At his best he was capable of the highest attainments. But he could not settle down to the drudgery of literary work and he found great difficulty in expressing himself on paper. His thoughts flowed too fast for the pen. But if he had a pencil and a sheet of paper he would spend hours in drawing the most complex sections and maps, which to the eye of a trained geologist were more expressive than many pages of printed description.

There was a complete absence of jealousy between them; so much so that they would freely criticise one another without the slightest fear of rupture. They were united also in an intense admiration for Lapworth. When in 1912 the three famous geologists visited Assynt together, and renewed acquaintance with the scene of their early triumphs, they would discuss how each point was gradually established beyond doubt, and it was interesting to observe how each of them insisted on giving all the credit to the others. This was a reflection of their generous minds; to both Peach and Horne it gave great pain to criticise anyone severely. Many a budding geologist who came to them for help found that their overflowing sympathies supplied all his deficiencies. And not the least valuable of their services to geology is the encouragement and training they gave to many beginners who were anxious to be put on the right path and to find support for their faltering footsteps.

J. S. F.



S. M. Paton

DIARMID NOËL PATON- 1859-1928.

DIARMID NOËL PATON died suddenly on September 30, 1928. He had been in failing health for some months, but no one had anticipated so sudden an ending on the very day on which he retired from the position which he had adorned so long, the Regius Chair of Physiology in the University of Glasgow.

He was the eldest son of Sir J. Noël Paton, the famous artist, and was born in Edinburgh in March, 1859. He received his early education at Edinburgh Academy, where he had as class mates W. Herdman, D'Arcy Thompson, and J. S. Haldane. Until he reached the age of 13 his whole bent had been towards drawing and painting, but contact with the budding zoologists, Herdman and Thompson, gave a new turn to his interests. They formed a club, called the Eureka Club, and spent their Saturdays hunting for fossils in the country round Edinburgh: later, inspired largely by White's "Natural History of Selborne," they included birds and butterflies in their interests. He was presented with his first microscope by his father in 1876 and spent much of his summer holidays in examining the fauna and flora both of the salt and fresh-water at Ardmay. Although art still attracted him strongly he finally decided, in 1877, to go to the University to study science and medicine, influenced as much, probably, by Matthews Duncan as by his early associates.

In his science course at Edinburgh University the men who influenced him most were Tait, Isaac Balfour and Crum Brown. In 1879 he began his first piece of research work, on the morphological significance of the seminiferous bracts of larch cones and on the course of the fibro-vascular bundles in the young cones. In 1881 he graduated B.Sc., being awarded the Baxter Scholarship, and a year later M.B., C.M. with first-class honours.

As regards his purely medical studies, Noël Paton was deeply interested in physiology from the outset. Anatomy, which he studied under Turner and Symington, interested him less. Pathology under Hamilton and the physiological side of therapeutics under Fraser he thoroughly enjoyed; but the final-year subjects did not attract him greatly.

Shortly after his graduation in medicine he went to Vienna, where he worked mainly at clinical subjects for six months; then went on to Paris, where he again devoted his attention to clinical work. In the early summer of 1883 he returned to Edinburgh to take up a house appointment, under Brackenridge, in the Edinburgh Royal Infirmary. Whilst a resident he worked up a case of Jacksonian epilepsy in the wards and this formed the matter of his first published paper.

In 1884 he was awarded a Biological Fellowship in the physiological department of the University of Edinburgh, under Rutherford, and began work on

urea formation and bile secretion. This work he eventually expanded into a thesis which he lodged for the degree of M.D. He obtained this degree with first-class honours and was awarded a gold medal for his thesis. About this time he was offered an assistantship by Underhill and he "put up his plate." He was also appointed physician to one of the City Dispensaries and a clinical tutor at the Royal Infirmary.

When, in 1886, the lectureship in Physiology at Surgeons' Hall became vacant Noël Paton was appointed, and two years later he succeeded Sims Woodhead as Superintendent of the Research Laboratory of the Royal College of Physicians, Edinburgh. He was now able to give up practice and devote his whole time to research work and teaching. He had opportunities at the College of Physicians and these he utilised to the full. At that time the majority of the younger workers in Edinburgh turned to this laboratory for encouragement, and this Noël Paton, ably seconded by Sir John Batty Tuke, then the Curator, freely gave. During his tenure of office the output from the laboratory, in all fields of medical research, was unequalled in Britain, as is clearly manifest from the volumes of the laboratory reports.

The subjects to which Noël Paton devoted his attention at this time lay mainly in the field of chemical physiology; indeed it may be claimed that he was the first physiologist in this country to devote serious study to the problems of metabolism. His work on urea and the end products of protein metabolism, on hepatic glycogenesis, on the physiology of the carbohydrates, on the nature of muscular energy and on hæmatological problems was both interesting and critical. In 1898 there appeared a valuable report, issued by the Fishery Board for Scotland, on the "Life History of the Salmon in Fresh Water." This consisted of seventeen papers by Noël Paton and his collaborators in the Royal College of Physicians' laboratory.

The problem of dietaries always interested him. In 1900, working under the auspices of the Town Council of the City of Edinburgh, in conjunction with Dr. J. C. Dunlop and Miss Elsie Inglis, he published the first of his studies on dietetic subjects, "The Diet of the Labouring Classes in Edinburgh." This was the first serious enquiry, along modern lines, into mass dietary questions in this country. He also, in 1903, published a report, through the India Office, on the nutritive value of certain "famine foodstuffs," and later, in 1905, a study of vegetarian diets.

In 1906, on the resignation of Prof. J. G. MacKendrick, he was appointed to the Regius Chair of Physiology in the University of Glasgow. He took over in Glasgow a new laboratory planned and built under the direction of his predecessor, but still awaiting equipment. Noël Paton devoted himself, heart and soul, to making his new laboratories as complete and up-to-date as possible. As soon as the equipment of the department was finished he showed the untiring energy which characterised his Edinburgh days in initiating and stimu-

lating research work. His own early work in Glasgow was mainly devoted to the study of endocrine function, a field in which he was a pioneer worker. As the result of these studies he published, in 1913, an interesting and stimulating book on the "Nervous and Chemical Regulators of Metabolism." This was followed by an intensive study of the function of the parathyroid, or as Noël Paton preferred to call them, the parathyreoid, glands. Whether his final conclusions be accepted or no, these investigations are most complete and thorough and will always have to be reckoned with in any further study of these glands.

Concurrently with his endocrine studies Noël Paton directed a series of investigations on dietary problems, for the most part studies of the diet of the very poor, which culminated in the elaborate study carried out in conjunction with Prof. L. Findlay on the relation of poverty, nutrition and growth. The relative effects of overcrowding, underfeeding and maternal care, or lack of care, were investigated in a way never before attempted. The result of their investigation was to show that environment, in the broadest sense of the term, is less important than the human factor. This report was published by the Medical Research Council. Noël Paton also collaborated with Prof. Findlay and others in a series of studies on "Rickets" in which the popular vitamin hypothesis was vigorously combated.

Noël Paton's interest in the factors involved in heredity had been aroused by his endocrine investigations and had been further stimulated by his sociological enquiries. He now took up this problem, and as a result published, in 1926, "The Physiology of the Continuity of Life," in which he attacked many of the accepted views. Noël Paton hated *ex cathedra* teaching, maintaining that so-called authoritative text-books have always played a great part in hindering the advance of science. A motto he admired was "*Nullius addictus jurare in verba magistri*." He tried to keep an open mind himself and he demanded from his students the exercise of their own judgment on the validity of the data he placed before them.

The last work on which he was engaged was a re-examination of the factors involved in the adjustment of posture and postural apnoea in the duck and swan. He published in all between eighty and ninety papers, as well as text-books of physiology for medical and veterinary students.

Enthusiastic as Noël Paton was as a research worker, he was equally enthusiastic as a teacher. It may be said without fear of contradiction that he was one of the last "all round" professors of physiology in this country. He was interested in physiology as a whole and he taught it as a whole. He was, moreover, really interested in teaching and was constantly endeavouring, up to the very last, to make his course well-balanced, stimulating and fresh. He never attempted to make his students academic physiologists, as he never forgot that the great majority of them would eventually become general

practitioners of medicine. He tried to instil into them, so far as he was able, physiological principles which would enable them to tackle in a scientific fashion their clinical problems. He retained a vivid memory of his own difficulties in early days when he was a clinical tutor. He was an admirable expositor, clear, succinct and thorough. Few who saw them will ever forget the masterly blackboard sketches with which he illustrated his lectures. He loved, too, to devise diagrams which would at once be simple, illuminating and yet comprehensive.

He found time to serve on many committees and boards. He was a member of the Royal Commission on Salmon Fisheries in 1900; of the Food Committee (War) of the Royal Society and took an active part in its proceedings; of the Advisory Committee on Agricultural Science of the Development Commission; of the Medical Research Council from 1918 to 1923. He was elected a Fellow of the Royal Society in 1914 and served on the Council from 1922 to 1924. He was elected a Fellow of the Royal Society of Edinburgh in 1886; was Vice-President from 1918 to 1921 and served three times on the Council (1894-97, 1904-06, 1909-12). He became a Fellow of the Royal College of Physicians of Edinburgh in 1886. In 1919 he received the honorary degree of LL.D. from his Alma Mater.

As a man he was tall, alert, somewhat spare in build and handsome in face. Beneath a rather formal, sometimes an aloof, manner Noël Paton hid a very warm, sensitive and kindly heart. He was strong in his likes and dislikes and did not hesitate to express them in uncompromising fashion. His own impatience of delay or of slowness of response often, it is to be feared, defeated his own ends. And yet despite his irritability no one could be kinder if once his heart were reached. No case of real difficulty or hardship left him unmoved. As a colleague he was ever anxious to do his share, he acknowledged help given by co-workers freely, and he was equally ready and willing to contribute from the wealth of his experience.

His interests outside physiology were keen, although not wide. He had a sound knowledge, general perhaps rather than particular, of both literature and art. His appreciation of outdoor life was acute and he gained his real solace from nature rather than from men. Angling, in his later years, was his true relaxation. His enthusiasm for his beloved Tweed, by whose banks he died, was infectious. Life held no greater joy for him than a day spent on its banks rod in hand. Wet or dry, with good basket or poor, he always enjoyed his beautiful stretch of water between Drumelzier Haugh and Burnfoot.

But at work or on holiday the problems of life and its mysteries were constantly with him. He lived for science. Science was to him something even more than the most engrossing pursuit; it was a religion, whose teachings he accepted unfalteringly,

E. P. C.



Char. L. Gomes

SIR CHARLES SISSMORE TOMES— 1846–1928.

SIR CHARLES TOMES died on October 24, 1928, at his home, Mannington Hall, Aylsham, Norfolk, at the age of 82. Born in London on June 6, 1846, he was the son of Sir John Toms, F.R.S., and his wife, Jane Sibley. He came of the family of Toms, or Tomes, which had been settled at Long Marston, in Gloucestershire, since the reign of Richard II.

He was educated at Radley during the Wardenship of the Rev. W. Sewell and attained distinction as an oarsman, rowing in the Radley Eight in 1863. On leaving Radley he went up to Oxford and entered Christ Church as a commoner. He rowed in the Oxford Trial Eights in 1865. Even in his old age he still retained the tall, spare, handsome figure of the typical rowing man.

The obituary notice of his father, which he contributed to the *Proceedings* (Vol. 59, 1895, p. xiii), is the record of a career of pioneer work singularly like that of his own life-story. When John Toms was a student in London, Dr. R. E. Grant, Professor of Comparative Anatomy and Zoology at University College, gave him the tooth of a kangaroo and suggested that he should examine it. This was the origin of the important memoir on the teeth of marsupials, which was published in the *Philosophical Transactions* in 1849 (p. 403) in the form of a letter to Dr. Grant. It begins with a declaration of disagreement with the statement made by Owen, in his *Odontography*, that "the dentine, enamel and cement of the teeth of marsupial animals present the usual microscopic characters of these tissues in mammalia," and an insistence upon the value of dental histology for the display of Ordinal characters and for affording indications of affinity, a claim which, in the hands of his son and his pupils, was destined to lead to results of far-reaching significance.

The papers which were published in the same volume of the *Philosophical Transactions* include one by W. C. Williamson on the microscopic structure of the dermal teeth of some ganoid and placoid fishes, Thomas Henry Huxley's memoir on the Medusæ, and Dr. Mantell's monograph on *Iguanodon*. John Toms's next memoir in the *Philosophical Transactions* was presented by William Bowman.

Reference is made to these names to suggest the environment in which Charles Toms was brought up. His father was on intimate terms with the leading biologists of the time, so that Charles was constantly meeting these great men and hearing them discuss the new movement in biology then beginning to gain momentum. But above all there was the influence of Sharpey, at University College. Sharpey was the prime mover in the cultiva-

tion of histology and the development of practical physiology in this country, who sent his missionaries, Michael Foster, Burdon Sanderson, Gaskell and Sharpey-Schafer to carry the new discipline to a series of British Universities, and also Newell Martin to do a similar service in America and play an important part in building up the biological school at Johns Hopkins University. But, in addition, he stimulated John Tomes's interest in histology, and this was perhaps the chief factor in building up the essentially British science of odontology. Charles Tomes followed in the footsteps of his father, and when he went to Oxford he came under the influence of one of Sharpey's pupils, and became for a time a demonstrator of physiology to Burdon Sanderson.

But before this he studied anatomy under Prof. Rolleston and from him acquired a wide outlook in biology. When we recall the influences of his early environment it is not surprising that as his special subject he chose histology, the teaching of which had recently been inaugurated in Oxford. Hence, as soon as he had completed his professional training, he was ready to co-operate with his father in his well-known researches in dental histology, with which the name of Tomes will always be associated.

In 1866 he gained the only First Class awarded that year in the School of Natural Science. After graduating at Oxford Tomes became a student of medicine at the Middlesex Hospital, where his father was the dental surgeon. After obtaining high distinction in his school he qualified in 1869 for the diploma of Membership of the Royal College of Surgeons and the Licentiate'ship in Dental Surgery.

During the first four years of Charles Tomes's life his father was engaged upon those important pioneer researches on the comparative histology of teeth, published in the *Philosophical Transactions* in 1849 and 1850, concerning which many years afterwards Prof. Waldeyer, of Berlin, wrote: "Sir John Tomes opened the way to a correct interpretation of dentine." He might also have added, with equal justice, "of the enamel." During the most impressionable years of his boyhood Charles Tomes was living in the atmosphere of scientific investigation. During his undergraduate years at Oxford Darwin's *Origin of Species* was published; and in the controversies that developed around it his father's most intimate friends played a conspicuous part. Moreover, the subject of his father's investigations suddenly acquired the most fundamental importance, because odontology affords evidence of outstanding significance for the solution of problems of affinities and of phylogeny. Hence it was not surprising that as soon as Charles Tomes entered on the practice of his profession he also began original investigations on the comparative histology of teeth, and that he soon began to cultivate the wider field of odontology and collect evidences of the relationships of vertebrate animals. The monograph on the development of the teeth of amphibia and reptiles, which was published in the *Philosophical Transactions* in 1876, was

regarded as a work of such distinction that Tomes was elected to the Fellowship of the Society in 1878 at the early age of 31.

Although he was busily engaged in the practice of dentistry, at first in association with his father in Cavendish Square, and was in succession lecturer on anatomy and physiology, surgeon and consulting surgeon at the Dental Hospital, he found time during more than thirty years to publish a series of important monographs on the morphology, microscopic structure and development of teeth in a wide range of vertebrate animals. His researches on the development of enamel, especially those published in the *Philosophical Transactions* for 1898, extending his father's pioneer work, laid the foundations of this difficult but very important field of investigation. His research on the teeth of Creodonts in 1906 played a significant part in emphasising the possibility of eliciting information regarding the affinities of long extinct fossil animals from the histology of their teeth, a line of attack on problems of vertebrate phylogeny, which, in the hands of his pupil, Mr. Thornton Carter and others, has been conspicuously fruitful in recent years. He also carried out researches on the growth of the jaw and on the chemical components of enamel.

In all his work Tomes was a very careful and accurate observer. He was very cautious in the inferences he drew from his observations. Hence a great part of his published researches, even the pioneer work done more than half a century ago, has not merely stood the test of time, but still remains the best account of certain aspects of dental histology.

Here again his account of the qualities of his father's work applies with equal appropriateness and cogency to his own :—

“ His work has stood the test of time, and, like that of his friend Bowman, remains to this day in all essentials unshaken. It is very remarkable that the work of these early investigators, working with instruments that nowadays would be thought very imperfect, and with the methods of histological research all in their infancy, were so accurate and sound as time has proved them to have been.”

The greatness of Charles Tomes's achievement in building up our knowledge of the comparative histology of teeth is perhaps surpassed by the influence exerted by his *Manual of Dental Anatomy*, the first edition of which was issued in 1876. Before then other books on dental anatomy had been published, but none of them were so comprehensive as the book written by Tomes, dealing, as it did, with conspicuous care and thoroughness with the macroscopic and microscopic features of the teeth of man and vertebrates in general. Ever since its first appearance it has remained the standard text-book in English, which has passed through numerous editions and exerted a world-wide influence both on teaching and research in dental anatomy. Although

recent editions have been edited by other hands, the manual still preserves the stamp of its original author. After entrusting the revision of the book to others, who might have ampler leisure to deal with the rapidly-expanding literature of comparative anatomy and palæontology, he still maintained control of the section dealing with the teeth of man. In 1859 Sir John Tomes published a work on "Dental Surgery," the subsequent editions of which were edited with considerable additions by his son.

Charles Tomes's outstanding achievement in original research was his work as an odontologist and interpreter of the value of dental morphology as evidence of phylogeny. In the year 1825 F. Cuvier had published his treatise, "Des Dents des Mammifères considérées comme caractères zoologiques." This was followed in 1845 by Owen's "Odontology." In the later editions (1882, 1889, 1894 and 1898) of his *Manual of Dental Anatomy* Charles Tomes developed his chapter "On the Evolution of the Forms of Teeth," dealing with the theories of concrescence first advanced by Gervais in 1854 and elaborated by Kükenthal and by Röse; the cingulum theory; the theory of kinetogenesis; the multitubercular and the tritubercular theories. He developed and elucidated in the most masterly manner the various factors underlying the progressive changes in tooth form.

One who was a student in Tomes's class at the Dental Hospital of London in the year 1898-1899 tells the writer of this notice that the fascination of listening to the lecturer explaining the great principles of evolution as they are revealed in the teeth of vertebrates, amplifying his written work from the rich store of his unique knowledge, exercised a greater influence upon his mind than anything else in his professional training. At the close of a lecture Tomes would gather about him those who wished for further information, and with infinite patience and in the simplest language would explain any difficulties.

In spite of a natural diffidence and shyness he longed to impart to his students his own all-absorbing interest in Dental Anatomy. His method of criticism was so gentle and considerate that he was often able to give the necessary correction without letting the victim know he was being criticised. This spirit endeared him to his pupils and colleagues. One example will illustrate his method. A student, deeply in awe of a lecturer who was the world's greatest authority in his subject and the acknowledged leader of the dental profession, found himself alone with Tomes in the Museum of the Odontological Society for a *viva-voce* examination. Tomes talked for a little while and then said, "Do you know the dental formula of *Myrmecobius*?" "No, sir," replied the nervous student. "Neither do I," said Tomes! Then walking to a case, he took therefrom some wax models made by Röse to illustrate the successive stages of tooth development; he looked at one for a little time and then said, "Aren't they really beautiful?" More than a

quarter of a century later this student, to whom dental anatomy had become the absorbing interest of his life, received from Tomes, then an old if still very alert man, a letter containing many valuable suggestions as to the preparation of sections of friable material.

The true significance and importance of a man's work lies not so much in his own achievements and accomplishments, but in the extent and degree in which he becomes a source of inspiration to those who follow in his footsteps. John Tomes was the intimate and peer of Grant, Sharpey, Williamson, Mantell, Huxley, Quekett, Carpenter and Owen, together with all the leaders in biological and medical science. To his intellectual ability he added a skill in mechanical dexterity which amounted to genius, and made him not only the foremost dental surgeon of his day, but also a marvellous technician in the preparation of ground sections of teeth.

He accumulated a collection covering a wide range of mammals and other vertebrates. This collection, with like skill, was largely augmented by Charles Tomes; and an unfortunate accident having impaired his vision to such an extent as to terminate his microscopical researches, this unique material was presented by him to the Royal College of Surgeons, where it is now one of its most valued and valuable possessions.

Even when this collection had passed out of his possession Charles Tomes still maintained a deep interest in the application of dental histology to the problems of phylogeny—a field in which his father's memoirs on "The Structure of the Enamel in the Rodentia," and his own brilliant contribution on "The Structure of the Enamel in the Creodonts," had led Thornton Carter to undertake the investigation of the structure and pattern of the Enamel as a test of affinity in other Orders of Mammals. (*Proc. Zool. Soc.*, 1922.)

In addition to his professional and scientific work, Sir Charles Tomes was an examiner in dental surgery for many years at the Royal College of Surgeons, the Fellowship of which was conferred on him in 1889, as had previously happened in the case of his father. He served as the Crown nominee on the General Medical Council from 1899 to 1920, and for fifteen years of this period he was the Treasurer for the Council and on one occasion the acting President. During the war he served as Chairman of the Norfolk and Norwich Hospital, and was inspector for the Norfolk Red Cross Hospital. He was knighted in 1919, and ten years previously the University of Birmingham conferred upon him the honorary degree of LL.D. He was formerly the Surgeon-Dentist to the King.

During the last year of his father's life the members of the dental profession founded the John Tomes Prize to commemorate Sir John's great achievements, not merely as a scientific investigator and teacher, but for his public services. When he began to practise, dental surgery was little more than a trade: he made it an honoured profession. It was appropriate that the Council of the

Royal College of Surgeons, in making the first award in 1894, should have chosen Charles Tomes as the first recipient.

For many years he took an active part in the work of the Zoological Society of London, of which he was a Vice-President. He was not merely interested in comparative anatomy, but he also loved living creatures, and especially dogs. To the end of his life he continued to maintain a keen interest in the problems at which he had worked in his earlier days, and his opinions were still inspired by a breadth of view and accuracy of information as well as by a keenly critical spirit. He was a singularly open-minded man, and, unlike many addicted to criticism, never resented criticism himself, and was always ready frankly to admit points scored against him. Yet it can truly be said of him what he wrote of his father: "His methods, whether in scientific or public matters, were never controversial, and he was one of those few people able fully to retain the friendship and liking of those from whom he might have occasion to differ most strongly." (*Roy. Soc. Proc., loc. cit., p. xiv.*)

He was a skilful painter in both water and oil colours and exhibited at the Royal Academy under the name "Charles Sissmore." He was an enthusiastic fisherman and sportsman. His common sense and freedom from any sort of pettiness were qualities that, in at least as great a degree as his knowledge and critical ability, made his advice as valued by his friends as his services were useful to public bodies. For he was free from that pride in exercising his influence that hampers the best intentions of many men of ability when they dispense patronage.

In 1873 he married Lizzie Eno, daughter of Dr. Charles D. Cook, of Brooklyn, U.S.A., who, with their daughter, survives him.

A writer in the *British Medical Journal* (of November 3, 1928), one of his friends of long standing, expresses, with more authority and eloquence than the present writer commands, an appreciation of Sir Charles Tomes's personality:—

"After his retirement from active practice in Cavendish Square he settled in Park Crescent, where he and Lady Tomes enjoyed for many years the pleasure of entertaining his numerous appreciative friends—professional, musical and artistic—who delighted to discuss, if they did not always agree, with him, the prominent subjects of the day. His scientific trend of thought, tempered by a strong vein of common sense, often proved valuable in counsel as well as interesting in social life. Himself a fine water-colour artist, he was always willing to listen to criticism of his own or others' work, even from the point of view of the man in the street. To those privileged to share his holidays in the North and elsewhere, his keenness for sport with a gun and fishing-rod was infectious, but for him there were no 'off days.' When others

were inclined to rest from their self-imposed labours he would be away in search of a good subject for his next sketch.

“His artistic prevision led him in later years to seek out a home which should be a fit setting for the wealth of fine furniture, china, pictures and other works of art which he had from various sources accumulated. Such a setting he found in Norfolk in the old moated house known as Mannington Hall, near Aylsham. There for many years he was able to entertain friends, to enjoy and constantly to enrich the fine old garden, to join in the sport for which the county is famous, having from his childhood been a keen shot himself, and employing spare time in research into the history of Mannington Hall and its previous owners and occupants, of which he published an interesting account in pamphlet form. Upon the introduction of wireless telephony he at once entered into the study of its phenomena, and made countless experiments of his own with a view to improve reception, until his failing powers during the last few weeks brought them to an end. An indefatigable worker, whether in science or in sport, he will always be remembered by those who knew him best as a modest leader, a sympathetic colleague, and a staunch friend.”

In the work of compiling this notice the writer has had the valuable assistance of Miss Amy Tomes, J.P., who continues the association with University College begun by her grandfather (Sir John) by rendering valuable service as a member of the College Committee, of Mr. C. Forster Cooper, Director of the Zoological Museum, Cambridge, who was intimately associated with Sir Charles after his retirement to Norfolk, Dr. David Stewart, of the University of Manchester, who is doing valuable research in dental histology, and in particular to Mr. J. Thornton Carter, who, formerly a pupil, is now the scientific successor of Sir Charles Tomes in carrying on and developing the work which has made odontology perhaps the most important instrument of phylogenetic research.

G. E. S.

SIR HUGH KERR ANDERSON—1865–1928.

It will help those who come later to realise what Anderson meant to his generation if two simple facts are put on record, which I believe to be without precedent. The University of Oxford was formally represented at the service in Caius College Chapel, and the Vice-Chancellor of Cambridge University ordered a special Memorial Service to be held in Great St. Mary's Church. I do not recollect any event which stirred the University more deeply than did his death. There was a sense of personal loss. Anderson's personality, his capacity for friendship, was of such importance to his contemporaries as to make an apology for going outside a mere record of achievement unnecessary.

Hugh Kerr Anderson was born on July 6, 1865, at Frogna Park, Hampstead, of Scottish parentage. He died at the Master's Lodge, Caius College, on November 2, 1928. His grandfather, Dr. John Ford Anderson, a son of Alexander Anderson, surgeon in the Royal Navy, came of a family long settled in the Buchan hamlet which shelters under the castle of Inverugie, some few miles from Peterhead. John Ford Anderson married a daughter of James Skelton, merchant, shipowner, and shipbuilder, in Peterhead, at that time a port of some commercial standing as well as a centre of the whaling industry, and, dying at the early age of 28, left his widow with a family of two sons and two daughters, ill-provided for.

Of this family the second son, James Anderson, Sir Hugh's father, came to London in 1828 to seek his fortune, with little more than the proverbial five pounds in his pocket, and entered the shipping firm which to-day under the title of Anderson, Green & Company, are managers of the Orient Line of steamers. He married Eliza Murray, the daughter of Surgeon-General John Murray, a woman of great refinement, ready wit, and no small artistic sense, Sir Hugh being the third son of their family of seven daughters and four sons.

This summary record of Anderson's immediate forbears may not be irrelevant, in so far as it is possible, without any extravagance of fancy, to detect in it the influences whether of heredity or association which contributed to form in him traits of character, faculties, and tastes so diversified as seldom to be found in effective alliance. Through his father he derived his sobriety of outlook, with at times a strain of melancholy, his sound common-sense, his faculty for business, and devotion to duty; through his mother his sympathetic and sensitive temperament, a playful and at times almost puckish humour, and a love of the beautiful in all its forms. Through both he derived his interest in the science of medicine.

Anderson was educated partly at a private school and partly by tutors, until in 1880 he went to Harrow, being for his first year in Mr. Griffith's house



John K. Anderson

and subsequently in Mr. Hallam's. He was a competent classic. For more than a year before gaining the Sayer scholarship and leaving Harrow in 1884 he was a member of the sixth form under Dr. Butler.

Though he never contemplated desertion to the modern side—a relatively recent institution—it was even then very apparent that the master bias of his life was towards the study of the natural sciences. Of the strength of this bias it is sufficient evidence that it prevailed, despite the lure of sentiment and the material advantages which a career in his father's business held out to him. Had his election fallen otherwise than it did, there may be no doubt of the measure of success which his shrewdness, diplomatic abilities, *flair* for finance, and keen abiding interest in all that pertained to ships, would have commanded.

In the long series of letters, which after his mother's death he wrote almost daily to his father, there are slight glimpses of the development of his character, and in particular of his distrust of himself; a restraint upon the free exercise of his powers, which success in later life did much to dispel, though it did nothing to make him less critical of his work or less modest as to the results he achieved. Apart from these glimpses, the letters for the most part are concerned with the details of daily life, exclusively intimate and ephemeral in their interest. One passage, however, from his father's letters may be quoted here. It runs: "I would wish you so to live that you may be missed when God calls you hence." The wish was fulfilled, and the words might fittingly be his son's epitaph.

Anderson came to Caius in 1884 as a Classical Scholar. He held a Sayer Scholarship, which was founded by a Caian "for the Promotion of Classical Learning and Taste, from Harrow School, to be held at Caius College." He at once changed, however, to medicine, and, after taking a First Class in both parts of the Natural Sciences Tripos, went to St. Bartholomew's Hospital to complete his medical course. Somewhere in these years he attended Addenbrooke's Hospital, and cherished memories of the caustic and merciless way in which Sir George Humphry managed his classes. One of my recollections of these early years is of musical occasions when Anderson played the 'cello, and of him as an active member of the College Musical Society.

After taking his medical degree he returned to Cambridge to follow research in the Physiological Laboratory, where he was one of the third generation of Foster's men, the first exemplified by Gaskell and Langley; the second by Sherrington, Adami, Head and Rolleston; the third by Anderson, Rivers, Fletcher and Hopkins; and the fourth by Barcroft, Dale and Elliott.

From 1892 to 1905 there was a steady output of scientific papers, twenty-two in all. In 1905 the curtain fell, his election to the Royal Society in 1907 being in some sort a posthumous honour so far as his scientific life was concerned.

Anderson's career, therefore, is something of an enigma. In the first fifteen

years of his productive life he proved himself to be possessed of an acute scientific intellect and of an altogether rare quality as a teacher. Why did he not follow the ordinary course, occupy a Chair and become head of a school? Why did he suddenly cease actively to pursue scientific research to be the wise and kind counsellor in affairs, of whom it may be said in literal truth that "there is hardly any great project which has come to fruition in Cambridge for many years but he has been its most inspiring forwarder"? The answer lay in himself. Scientist, man of affairs, artist—he was all of these and the first two found obvious expression. The third, however, cannot be neglected since it informed and coloured the whole. These three struggled to possess him, and when the conflict was at its fiercest in the early '90's they tore him sorely.

The beginning of the eclipse, for eclipse it was, of a brilliant scientific career is to be found in a simple enough happening, when he first took his turn amongst the Fellows of his College to audit the College accounts. Finance was of the very make-up of his being, and from that first auditing followed activities which by 1903 had completely remodelled the College finances and so increased his influence within the College that when the headship fell vacant in 1912, the Fellows, practically without debate, elected him Master. No one was surprised save Anderson himself, and never in the history of any institution was there a more fortunate choice.

The date 1905 marks the time when even Anderson's immense industry was unable longer to bear the double burden of College affairs and active research. He did it is true and most fortunately for that generation continue the teaching of science until his election as Master in 1912.

In 1909 and again in 1912, when the Hall and Lodge were remodelled, the third side of his nature, his delicate and detailed sense of beauty, enabled him to give peculiar service to his College.

The University was not long in discovering the capacity for service which this junior Fellow of Caius possessed. In 1906 he became a member of the University Press Syndicate, in 1908 of the Financial Board, and in 1910 of the Council of the Senate. Thence onwards there is a record of ever widening public duties, of which the barest enumeration must suffice.

He became Chairman of the University Press Syndicate and in that capacity head of a great publishing house. In 1919 he was appointed a member of the Universities Commission, and in 1922 of the University of Cambridge Statutory Commission. His great influence upon the development of the medical school in its most critical years needs special mention.

Outside the University he was a Director of the London Life Assurance Company, a member of the Commonwealth Fellowship and Chairman of the Cambridge Waterworks Company. Had he acceded to the demands made upon him his activities would have been increased ten-fold, but he consciously reserved himself for the service of his College and of his University.

I reserve to the last an appointment which gave him peculiar pleasure. In 1922 he became a Governor of Harrow School, the appointment being made by the Royal Society. He had a great affection for his School, one symptom of which was an unfailing attendance at the Eton and Harrow match. In good fortune and ill he was always there and reduced to voicelessness at the close by his enthusiasm.

The published record of Anderson's scientific work by no means exhausts his contributions to science. His reach far exceeded his grasp and those who worked in Foster's laboratory at any time between 1892 and 1912 will recollect how fertile he was in ideas, how accessible and how stimulating it was to discuss work with him. No department of physiology seemed to come amiss. Contemporaries and pupils alike can bear testimony to the fact that in those years he was a great biologist.

I have mentioned his rare qualities as a teacher. Whilst he was lecturer in advanced histology, the subject had a character and uplift which it has not been possible to maintain. And everyone who had the good fortune to attend his classes, especially the small evening classes, or form one of the crowded audiences who listened to his lectures on the nervous system, will recollect how he never spared himself, how vividly original was his outlook and how totally impossible it was for him to reproduce in class or lecture clammy fragments of text-books.

Of his method of work the chief feature was a capacity for taking pains. Of the work on the Royal Commission "not a detail escaped him. He toiled day and often night, not only at documents presented to him, but in creating invaluable presentation of statistical and financial information." What a familiar picture those phrases call up to all who knew him intimately!

He was apprehensive in this, that he felt vividly the possibility and consequences of failure. Sensitive is perhaps the better word, sensitive to those small signs which point to success or failure, especially in dealing with people. Apprehensive and sensitive, yes: but, once the course of action was settled in his mind by the tremendous drill in detail to which he subjected all his ideas, no one could have been bolder.

It is comparatively easy to set down the outstanding features of his career and of his methods, but of the man it is more difficult to speak; and yet for his generation it was the man that counted. He was so gifted and so gentle, so capable of tendering help and so approachable by all who needed help. He was the well beloved, and on that sure foundation stood his great gifts of service. It is hard to write temperately of him as a personality—hard, because any form of words must fall so far short of the truth. W. B. H.

ANDERSON'S scientific work, viewed across the twenty four years since it lapsed, ranges itself primarily into two groups of papers, one, on the whole

an earlier, of joint authorship with Langley, his senior ; the other entirely Anderson's own.

Of the two groups, the former opened- -in the light of the partnership naturally enough -on a field then especially interesting Langley, the "sympathetic." This initial paper clinched by decisive demonstration the fact long questioned that the opening of the pupil under sympathetic stimulation is due to activation of a radially contractile dilatator. Nor was this the only paper in their series in which the two authors reverting to some old vexed question proceeded to settle it for good and all so far at least as concerned objective fact. A statement which sporadically cropped up, neglected and suspect because little consonant with current knowledge of reflex mechanism in general, was that with certain visceral ganglia their function included reflex actions. The two partners proceeded to look at first hand into the grounds for this assertion. They explored the facts systematically, sifting and adding to them. Their result was the discovery of a new and unexpected type of neural reaction ; and they furnished of it a penetrative and satisfying analysis. They thus placed on a basis of solid knowledge the "axon-reflex" as recognised by physiology to-day. There then followed the papers clearing up what had seemed the almost hopeless intricacies of the nerve-supply of the lower viscera, they replaced what had seemed chaos by a demonstrated scheme of fairly simple type -a two-link chain, pre-ganglionic with post-ganglionic neuron. From this work they passed with the special experience so gained to the problems of the limits of possible substitution of one kind of nerve-fibre for another after traumatic degeneration. They reached, in their customary convincing way, decisive results. The several sorts of efferent nerve-fibres springing directly from brain and spinal cord were shown to be all of them interchangeable one with another. But on the other hand such efferent fibres and their endings were found unable to replace and irreplaceable by afferent nerve-fibres and their endings. Further, the efferent fibres springing directly from spinal cord and brain were found uninterchangeable with those springing from the sympathetic ganglia.

Finally, this fertile partnership dealt with the problem, at that time much confused, of peripheral nerve-regeneration. Their paper placed on record a total failure in successive trials to obtain any evidence whatever of "auto-genetic" restitution of the distal portion of a cut nerve. In other words they found the restitution in all their experiments to be by outgrowth from the central side of the injury, *i.e.*, from the proximal stump of the cut nerve itself. The confidence felt in the observational power and judgment of the two researchers secured for their results, even when negative, an acceptance practically as contented as for positive data.

It can be said of the whole series of their papers that they furnished a succession of results which are an established part of neurology to-day. If the technique is somewhat that of a past era, that largely is so because in the hands

of its two adepts, such method, *sui generis* as it was, served its purpose and was, so to say, squeezed to its last drop. As much to the one of them as to the other its skill and fruitfulness belonged. Each, though assuredly widely different from his colleague in biological outlook had gifts of close resemblance to him in so far as concerns executive experimentation. As for directness of aim, minute precision, unswerving loyalty to observation, prodigality of patience for the reaching of a fact, in all these features the conjoint work reflects both its authors indistinguishably.

Turning to the papers emanating from Anderson working alone we are met almost at once by a title indicating a scope distinctly different from that of any of the conjoint work. The field entered is that of ontogenetic growth : the particular subject being the development, in their latter phases, of nerve-cells and their fibres. The enquiry consisted in experiments upon a possible trophic influence of one consecutive neuron upon another in the same neuron chain. Anderson found—and his paper frankly intimates his surprise at meeting the fact—that to cut off the terminal (post-ganglionic) neuron stunts the growth of the neighbour neuron above, but on the other hand the trophic condition of the terminal neuron below was not impaired by cutting off the neuron next above. In other words the direction of trophic influences seemed opposite to the direction of functional conductivity. Trophic nutrition and functional reaction thus appeared separable and even largely independent. The data of evidence were furnished with his usual conspicuous clearness and simplicity ; the phenomenon impressed him deeply and his discussion of it and its meaning strikes a note different from anything in the conjoint work.

He promised himself a return to the problem, but, at least in his published papers, he never got there ; from memories of him in those years I fancy, however, that he did subsequently do a good deal more on it, especially by following myelinization as an index to neuron development. Publication must have been prevented, probably, by other calls upon his time.

The above paper reaches probably the high-water mark of his research. Painstaking observations on the so-called paradoxical reactions of the pupil followed, but were, I think, less significant. The above-mentioned study and his conjoint work before cited assure him a place of permanent note in the story of biological enquiry of his time. The period, however, of his own first-hand activity in research was cut short by the call, which his public-spirited nature felt to be imperative, of other interests, those primarily of his College and his University. But he remained still keenly alive to what was going forward in research ; and where near enough to it he not rarely helped by consultation and critical encouragement. To withdraw from direct personal participation in it had been doubtless a wrench : in after years a regret in this respect would rise to his lips not rarely when talking with old friends.

C. S. S.

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